Food Research

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INSIDE SCIENCE

The Vital Story of VITAMIN C

by Science Writer

What does vitamin C do for human beings?

Medical research shows that it is essential in maintaining healthy bony tissue and for the formation of collagen which is the main supportive protein of skin, tendon, bone, cartilage and connective tissue. It is needed for vascular function and tissue respiration and in promoting the healing of wounds. In lactating women it has great importance in providing the infant with a sufficient amount of ascorbic acid to prevent scurvy, a disease to which very young babies are susceptible.

Much evidence is available in medical reports to show that a lack of this vital nutritive element can result in impaired health. And in those cases of frank scurvy which still occur, ascorbic acid is the specific medication.

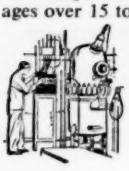
History. The history of vitamin C is fascinating. Scurvy was once the most prevalent disease in Europe. In the 18th Century the antiscorbutic value of fresh vegetables and citrus fruits was recognized.

Isolation. It was not until 1918-25 that almost pure ascorbic acid was concentrated from the lemon by Zilva and its important properties and molecular composition established. In 1927 he concluded that this so-called "reducing factor" and the antiscorbutic principle were closely related. In 1928, Szent-Györgyi, who started investigations in 1921, isolated a strong reducing compound from adrenal glands, oranges, cabbage, terming it "hexuronic acid."

Chemistry. The "reducing factor," "hexuronic acid," and vitamin C were all identified as the same in 1932 by various groups of workers. This was one year after C. G. King established the chemical identification of vitamin C.

Synthesis. In 1933 Reichstein and his colleagues in Switzerland successfully synthesized ascorbic acid. Almost simultaneously in England Haworth and associates accomplished the same. The Reichstein synthesis formed the basis for large scale commercial production by the world-famous firm of Hoffmann-La Roche.

Production. Following these discoveries came the establishment of manufacturing operations so that today daily production by the tons is the rule. The figures are really staggering. The amount produced weekly in the U. S. A. averages over 15 tons, equivalent to that contained in over 200 million oranges. It is being made synthetically at a very low cost which permits widespread use in many food and drug products. One kilo is sufficient to supply 92 people for one year with the minimum daily requirements set by the U. S. Food and Drug Administration.



Vitamin C made by the Roche process is identical chemically and in biological activity with Nature's own product. Because vitamins can be manufactured at a much lower cost than if they were extracted from natural sources they are widely used in dietary supplements.

Ascorbic acid is present in many foods. Nature supplies some with lavish amounts and stints on others. In fact, wide variations* have been found in the vitamin C content in varieties of the same fruit.

Deficiencies. Many surveys have shown widespread vitamin C deficiencies in the population. Physicians may call these subclinical, but they are deficiencies nevertheless.

Because of these situations—natural variation and widespread deficiencies—diet experts believe that it is desirable and in the public interest to standardize the vitamin C content of processed fruit and vegetable juices by the addition of enough pure crystalline ascorbic acid to make them consistently dependable sources of this necessary food element. Some of these juices are: tomato, grape, orange, grapefruit, pineapple.

Requirements. The U. S. Food & Drug Administration has set 30 milligrams as the adult minimum daily requirement of ascorbic acid. This daily intake is just about sufficient to prevent recognizable signs of vitamin C deficiencies although a subclinical or an unrecognized deficiency may exist. In view of this, the National Research Council has established 75 milligrams as the recommended daily male adult allowance and 70 mg. daily for female adults, which levels are supported by many controlled nutrition studies. However, the N. R. C. recommends 90 mg. daily for boys between 13 and 15, 100 mg. daily for boys between 16 and 20, and 150 mg. daily for lactating women.

This article is published in the interests of pharmaceutical manufacturers and food processors who make their good products better with essential, health-giving vitamin C. Reprints are freely available. Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 286 St. Paul Street, West; Montreal, Quebec.

*The table shows minimum and maximum levels of ascorbic acid in commercially canned juices. All figures are in milligrams per 100 grams of juice. Data from U. S. Department of Agriculture.

	Min.	*Max.*
Apple juice	0.2	3.6
Grape juice	0.0	4.7
Grapefruit juice	10.0	49.0
Orange juice	9.7	70.0
Pineapple juice	5.4	18.0
Tomato juice	2.5	32.0

THE ACTION OF LIPOXIDASE IN FROZEN RAW PEAS[a]

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(Manuscript received December 15, 1955)

Raw vegetables which are held in frozen storage for extended periods show a progressive decrease in quality, characterized by development of off-flavors and changes in the composition of lipid matter. One of the major changes occurring during the frozen storage of raw peas and other raw vegetables is the development of peroxides in the lipids *(2, 3)*. There was a loss of chlorophyll and carotene when frozen raw peas were held for periods from one to 6 years at –17.8° C. *(3, 8, 9)*. In contrast, blanched peas of the same lots stored under identical conditions did not show this loss of chlorophyll, or changes in composition of the lipid materials.

The enzyme lipoxidase has been postulated as being a causative agent for chlorophyll breakdown during frozen storage of raw peas *(8)*. The primary action of lipoxidase is to cause a peroxidation of the double bonds of certain unsaturated fatty acids. A concurrent action involves the oxidation of certain unsaturated plant pigments such as chlorophyll and carotene *(6)*.

To prove that lipoxidase is responsible for peroxidation of lipids and chlorophyll destruction during storage it is necessary, first, to demonstrate its presence in raw peas, and second, this enzyme must be isolated from raw peas, purified, and allowed to react with its substrates in such a manner that the effects of its action may be determined quantitatively.

This paper reports the demonstration of the presence of lipoxidase in frozen raw peas, its partial purification, and its action in bringing about the peroxidation of pea lipids and the destruction of chlorophyll in blanched peas.

MATERIALS AND METHODS

The frozen peas used in these studies were grown on Experiment Station plots and were processed as described earlier *(3)*. Certain lots of fresh peas were washed, sorted, frozen, and dried in a Stokes cabinet type freeze-drying unit with the heated plates at 27° C. The dried peas were stored in 30 pound tins at –17.8° C. until used. Fresh peas were purchased on the market as needed during the winter months.

Soybean lipoxidase was prepared from defatted soyflour,[b] and also was obtained commercially (Nutritional Biochemicals, Inc.). Pea lipoxidase was prepared from raw frozen peas and from lyophilized raw peas.

If lipoxidase was not denatured during drying of peas, then lyophilized raw peas would constitute a source of starting material available throughout the year for fractionation studies. Preliminary studies concerned with the use of lyophilized raw peas as a source of lipase had indicated that very little deterioration in the lipid fraction was encountered during storage at –17.8° C. Accordingly, samples of raw Perfected

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[a] Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 1009.

[b] Kindly supplied by Archer-Daniels-Midland Co., Minneapolis, Minn.

Freezer peas of the 1954 harvest, which had been lyophilized in the Stokes Freeze Dryer and stored at –17.8° C., were tested for lipoxidase activity using linoleic acid as substrate in the manometric method described below. The lipoxidase activity of lyophilized peas was roughly 60% that of the frozen raw peas on a comparable basis. Lyophilized peas could therefore be used instead of frozen raw peas as source material for lipoxidase fractionation.

Various combinations of centrifugation, dialysis, ammonium sulfate fractionation, and lyophilization were employed in order to attain maximum enrichment of specific activity with minimum loss of total activity. The activity of all fractions was checked and calculated on a dry weight basis. The lipoxidase was enriched 40.6 fold over that present in lyophilized raw peas. This preparation retained 75% of its activity after lyophilization and one month's storage at –17.8° C.

Lipoxidase activity was determined manometrically by the method of Süllmann *(7)* by measuring oxygen uptake of a linoleic acid emulsion in water in the Warburg respirometer at 30° C. Controls containing enzyme and no substrate, as well as autoxidation controls containing substrate and no enzyme, were included with every determination of lipoxidase activity.

In the studies of peroxidation of pea lipids certain samples of peas were lyophilized and extracted with ethyl ether in Soxhlet extractors as described earlier *(3)*. For other samples a different extraction technique was employed. Two-hundred gram samples of frozen peas were blended at top speed in the Waring blender with an equal weight of saturated NaCl solution. The resultant mixture was extracted 4 times with 500 ml. portions of peroxide-free ethyl ether in a four liter separatory funnel. Emulsions formed at this stage, even in the presence of saturated NaCl, and centrifugation of the mixture was required to break these emulsions. The mixtures were centrifuged for 10 minutes at 2000 r.p.m. in an International No. 2 centrifuge in 250 ml. Pyrex centrifuge bottles, equipped with rubber caps. The ether layers were decanted, combined, and the ether removed by evaporation under reduced pressure.

Determinations of peroxide numbers were made on ether-extracted lipid matter using the micromethod described by Lee, and Lee and Wagenknecht *(2, 3)*.

Total solids and chlorophyll were determined as described earlier *(3)*.

RESULTS AND DISCUSSION

When raw Thomas Laxton peas were lyophilized after 5 years' storage at –17.8° C., and extracted with ether in a Soxhlet extractor, the lipids showed evidence of mild peroxidation. However, when samples of these raw peas were blended with an equal weight of water or saturated NaCl solution for 5 minutes in a Waring blender, and the lipid matter extracted from this slurry with ether in a separatory funnel, relatively large peroxide numbers were obtained. Samples of blanched peas from the same lot showed no evidence of peroxide formation when analyzed by either of the above techniques.

Similar results were obtained with raw and blanched peas of another variety, Topper, after prolonged storage at –17.8° C. Frozen raw Thomas Laxton peas after 2 months' storage at –17.8° C., analyzed in the same manner, showed a similar peroxidation of lipid matter after blending and extraction with ether in the separatory funnel. However, no peroxides had as yet accumulated in the stored material (Table 1).

Storage of the peas was not an essential requirement in order to demonstrate the peroxidation of pea lipid. This was shown by blending fresh raw and also blanched market peas, extracting the lipids with ether in a separatory funnel, and analyzing the ether-extracted lipids for peroxide numbers. The peroxide number of the lipid from the raw peas was 251.3; that of the blanched peas was zero.

TABLE 1

Peroxidation of pea lipid matter during frozen storage and as a result of blending raw peas in the Waring blender

Variety	Storage time at −17.8° C.	Condition	Peroxide number[1]	
			Lyophilized material Soxhlet-extracted	Blended material extracted in separatory funnel
Thomas Laxton	5 yr.	Raw	24.0 (22.0-27.3)	408.3 (323.4-585.9)
Thomas Laxton	5 yr.	Blanched	0	0
Thomas Laxton	2 mo.	Raw	0	419.0
Thomas Laxton	2 mo.	Blanched	0	0
Topper	6 yr.	Raw	12.4	519.6
Topper	7 yr.	Blanched	0	.....

[1] Averages and extreme values, the latter in parentheses.

It was believed that the peroxidation of lipids observed in fresh and frozen raw peas was mediated by the enzyme lipoxidase.

No peroxide formation resulting from lipoxidase action was evident in aqueous phosphate buffer extracts of frozen fresh raw Thomas Laxton peas, either immediately after grinding the peas in a glass mortar with phosphate buffer, pH 8.0, or after 20 hours' incubation at 30° C. The lipids extracted with ether from these buffer extracts, likewise failed to show positive peroxide numbers. Such passive mixing of enzyme and substrate apparently was unfavorable for proper enzyme action. The vigorous agitation and aeration provided by the Waring blender apparently were necessary to achieve favorable conditions for enzyme action and resultant peroxide formation.

The optimum blending time for maximum peroxidation of the pea lipid is about 5 minutes. Shorter times did not allow for completeness of reaction, and during longer blending times, the peroxides were apparently partially used up. Blending in an atmosphere of oxygen did not produce effects much different from those obtained by blending in air. Blending of raw peas in an atmosphere of nitrogen caused a marked reduction, but not complete suppression, of peroxide formation in the lipid matter.

That lipoxidase does act oxidatively upon chlorophyll was shown by allowing an active preparation of soybean lipoxidase to act upon blanched peas. Soybeans are the richest known source of plant lipoxidase. A 1% aqueous suspension of defatted soyflour was used as a source material.

Five 100 g. lots of blanched Thomas Laxton peas which had been in storage 6 months at −17.8° C. were allowed to thaw at room temperature. Samples of 100 g. of these peas were blended with 100 ml. of the soyflour lipoxidase preparations in the Waring blender for 5 minutes. Prior to blending 0.1 g. $CaCO_3$ was added to retard chlorophyll destruction. Controls were run in a similar manner in which 100 ml. of distilled water was used in place of the soyflour lipoxidase preparation. Twenty gram samples of the resultant slurries were then analyzed for chlorophyll content by the customary procedure *(3)*. Total solids determinations were included, in order that chlorophyll content might be expressed on a dry weight basis.

This crude preparation of soyflour lipoxidase was capable of destroying 27% of the total chlorophyll of the blanched peas in only 5 minutes reaction time.

A purification of soyflour lipoxidase by ammonium sulfate fractionation was effected using the methods of Balls, Axelrod, and Kies *(1)*. Several active preparations were obtained which when blended with blanched Thomas Laxton peas caused chlorophyll destruction of from 30% in 5 minutes up to 40% when the blended mixtures were allowed to react for 2 hours prior to the acetone extraction step.

The activity of commercially obtained lipoxidase was tested using the manometric assay and also for chlorophyll destruction in blanched peas (as outlined above).

In the manometric assay, when used at a concentration of 0.5 mg. per ml. the commercial lipoxidase gave a $Q_{O_2}^{10c}$ of 702, or about twice the greatest activity obtained from lyophilized raw peas. The chlorophyll destruction data are given in Table 2.

TABLE 2

Action of commercial lipoxidase on chlorophyll in blanched Thomas Laxton peas

Sample	Time and temperature of storage after blending	Chlorophyll		
		mg./100 g. fresh wt.	% of control	% destroyed
1. Water control	0 min., room temp.	9.91	100	.....
2. 40 mg. lipoxidase	0 min., room temp.	7.86	79.3	20.7
3. 80 mg. lipoxidase	0 min., room temp.	7.47	75.4	24.6
4. Same as 1	60 min., 37° C.	9.01	100	.....
5. Same as 2	60 min., 37° C.	6.70	73.5	26.5
6. Same as 3	60 min., 37° C.	5.48	60.9	39.1

The percentage of chlorophyll destroyed due to lipoxidase action at room temperature was of the same magnitude as that previously obtained with crude soyflour lipoxidase. This destruction of chlorophyll was increased when the reaction mixtures were incubated at 37° C.

The mixtures of peas and lipoxidase which had reacted at room temperature and appropriate water controls were lyophilized and the crude matter was extracted therefrom with ethyl ether. Peroxide number determinations were run on these crude lipid samples. The lipid from the water control sample showed zero peroxide number; the lipid from lipoxidase-treated samples showed average peroxide numbers of 7.9 and 18.4, corresponding to 40 and 80 mg. of lipoxidase in the reaction mixtures.

Thus the two major actions of lipoxidase had been demonstrated using pea lipid as substrate; namely, chlorophyll destruction and peroxidation of the lipid material.

Lipoxidase activity has been demonstrated in the dried seeds of Thomas Laxton peas *(4)* and recently has been demonstrated in fresh peas *(5)*.

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[c] $Q_{O_2}^{10}$ = μl. oxygen uptake per mg. dry weight of tissue per hour, by extrapolation from values obtained after 10 min. reaction time.

The next step was the extraction and purification of lipoxidase from frozen raw Thomas Laxton peas, for it was during frozen storage of raw peas that the manifestations of possible lipoxidase action had first been noted *(3)*. A fractionation scheme employing ammonium sulfate precipitation and dialysis was used in the purification of lipoxidase from raw Thomas Laxton peas which had been held in storage for 6 months at –17.8° C. Preliminary investigation had revealed that an active lipoxidase was present in the raw peas after this period of frozen storage. The over-all purification of the enzyme using this fractionation procedure was threefold, a rather meager enrichment of the enzyme.

The action of the partially purified raw pea lipoxidase on chlorophyll in blanched Thomas Laxton peas was investigated. Two 100 g. lots of blanched Thomas Laxton peas which had been held in storage 6 months at –17.8° C. and which were from the same original lot of raw peas used to prepare the lipoxidase, were allowed to thaw partially at room temperature. One-hundred gram samples of blanched peas were blended with 100 ml. of water [or with 100 ml. of raw pea lipoxidase preparation (11.5 ml.) of purified lipoxidase made up to 100 ml. with distilled water] in the Waring blender for 5 minutes. Samples of the resultant slurries were then analyzed for chlorophyll content and total solids.

The lipoxidase actually added in this experiment represented but 39.5% of the total material originally present in the 100 g. of raw peas and contained but 10.4% of the original lipoxidase activity. However, even this relatively small amount of pea lipoxidase was capable of destroying 3.1% of the chlorophyll in 100 g. of blanched peas in 5 minutes and up to 10% of the chlorophyll in one hour's reaction time. Thus it may definitely be concluded that the native lipoxidase of frozen raw peas can bring about the destruction of chlorophyll in other peas. This phenomenon may well account for a large portion of the chlorophyll loss sustained when raw peas are held in frozen storage for protracted periods of time.

SUMMARY

Extensive peroxidation of the lipid matter of raw peas can be achieved by blending the peas with water in the Waring blender for 5 minutes. This phenomenon has been demonstrated in fresh peas and in frozen raw peas. Blanched peas do not form peroxides under these conditions.

The presence of lipoxidase in fresh and frozen raw peas has been established, and the enzyme has been obtained in partially purified form from fresh and lyophilized raw peas.

Lipoxidase is capable of causing peroxidation of the lipids and also the destruction of chlorophyll in blanched peas.

The reactions through which lipoxidase may contribute to off-flavor formation and other changes in quality of frozen raw peas have been demonstrated.

Acknowledgment

The authors are indebted to Miss Rosemary Graham for her assistance in these studies.

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OBJECTIVE TESTS APPLICABLE TO QUALITY STUDIES OF ICE STORED SHRIMP

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(Manuscript received April 26, 1956)

Objective quality tests are urgently needed by the shrimp industry to permit distribution and processing of individual catches of shrimp according to their expected storage life.

Heretofore, organoleptic measurements of quality have been used almost exclusively for purchasing and grading shrimp products at various stages during their storage life. These tests are very useful for rapid examination of such products. Unfortunately, they are based on personal judgment and consequently result in numerous subjective differences which lead to disagreement in qualification of individual samples. The subjective differences render it desirable to supplement organoleptic testing with a more exact analysis based upon changes of certain constituents in shrimp during storage.

Thorough organoleptic studies on shrimp quality *(12)* showed that definite taste changes occurred during ice storage, indicating that palatability and flavor change only slightly during the first few days of ice storage. The flavor is described as characteristic of "fresh" shrimp and is slightly sweet. This phase is followed by a period during which the shrimp no longer have this characteristic sweet flavor but instead are "flat" tasting. During this time, no off flavors associated with spoilage are noted. This second period is followed by another sharp change in flavor which denotes onset of spoilage. Repeated organoleptic studies with ice-stored shrimp by experienced taste panel personnel substantiated these three general flavor changes.

Since these results show definite taste phases during ice storage of shrimp, useful objective tests must discern the three major taste changes.

An ideal objective test would be one which would accurately and reproducibly give a numerical value of quality. The shrimp industry desires a test which can be correlated directly with the number of days shrimp have been in ice storage. It is rather doubtful that such a test can be perfected because physical and chemical changes are dependent not only on elapsed time of storage, but also upon treatment prior to storage and the environment from which the shrimp were obtained initially.

Any measure of definite chemical changes occurring in shrimp during storage under various conditions can be used to determine relative quality provided appropriate comparisons are made. Except for several tests described below it is unlikely that specific chemical concentrations can be assigned as a means of qualifying a particular sample of shrimp since the initial content will differ depending on such facts as age, size, specie or living environment. If analysis shows that a constituent such as ammonia nitrogen increases sharply after a specific storage time, and that slightly

different treatment or storage prolongs the time before this increase is encountered, it can be assumed that the latter handling or storage conditions were superior. Most of the following tests are measurements of relative quality of shrimp stored under specific conditions. However, it must be emphasized that physical or chemical treatment of samples can prevent accurate analysis and invalidate these methods.

Fish spoilage is derived from metabolic changes in tissue (autolytic changes), autoxidative processes and bacterial activity. In the past, several reviews have appeared in the literature concerning biochemical changes in fish during ice storage *(1, 24, 25, 29)*, and these contain information which is generally applicable to changes in ice stored shrimp. Most investigators believe that spoilage of ice stored fish is caused mainly by bacterial enzymes, but autolytic changes are definitely important *(21)*. A sudden increase in bacterial load is often taken to indicate onset of spoilage in fish and comparisons with organoleptic changes in shrimp show the same relationship exists *(10)*. In most cases, a bacterial count of 10×10^6 or more indicated spoilage regardless of the storage conditions of this product. Bacterial counts alone are unreliable in differentiation of various degradation stages in quality of shrimp but are very useful in conjunction with some of the other tests mentioned in this paper. Recently developed tests in this laboratory *(22)* are useful for simple and rapid approximation of the number of bacteria in shrimp and other seafoods. These tests require only simple equipment and usually can be completed in less than 3 hours.

Some investigators consider tests for ammonia unreliable as an indicator of bacterial spoilage of fish *(24, 25)*, and there is no general agreement concerning ammonia content as a quality index for these products. Studies conducted in this laboratory *(11)*, however, show that Nessler nitrogen increases slightly with the onset of spoilage and then rapidly as spoilage progresses. No definite spoilage index for Nessler nitrogen can be quoted at this time, but further study may reveal such a value for this constituent. These data indicate a rapid degradation of nitrogen containing components as spoilage begins. Trimethylamine nitrogen content and volatile acids of shrimp increase coincident with the onset of spoilage indicated by organoleptic tests and by bacterial counts *(12)*. Numerous experiments in this laboratory have substantiated these general results. A trimethylamine nitrogen value of 1.5 mg./100 g. unshelled, headless shrimp has tentatively been specified as an objective index of spoiled shrimp *(12)*, and the results of further experiments indicate that this value is reliable. No definite figure can be cited for volatile acids as an objective measure of spoiled shrimp since the concentrations of these materials are highly variable in different shrimp. The changes noted above were expected since both trimethylamine nitrogen and volatile acids are metabolic breakdown products of bacteria in seafoods *(7, 29)*.

Amino nitrogen decreases rapidly in ice-stored shrimp during the prime quality period, remains relatively constant during the second quality phase and then decreases rapidly during spoilage *(10)*. Beatty and Collins *(4)* also found that amino nitrogen content of press cod fish juice decreased during storage and that the decrease approximately equaled bacterial deamination. Opposite results for amino nitrogen content were reported

by Campbell and Williams *(6)* for shrimp, and by Sigurdsson *(25)* for herring; both containing increases in amino nitrogen during ice storage. These differences can possibly be explained on the basis of different bacterial flora in various seafoods resulting in different protein degradation products, although it has been shown that amino nitrogen content of fish depends on the autolytic changes in fish flesh as well as the chemical composition of the flesh *(21)*. Another possibility is the rapid association of amino groups with denatured protein molecules masking them from detection by the procedure used.

During the past 3 years, numerous chemical, physical and microbiological tests have been used in attempting to objectively distinguish shrimp in the various organoleptically discernible quality phases, as well as to compare the relative quality of shrimp handled and stored under varying conditions. A large number of these were of no value as objective tests and hence were discarded. The acceptable tests for ice stored shrimp can be classified into three categories: I. Those indicating definite changes in prime quality; II. Those which signify onset of spoilage; and III. Other tests for determining relative quality but which do not signify onset of spoilage or indicate definite changes in prime quality. All tests in these classes can be used for relative comparison of different shrimp samples.

Table 1 shows the Tests studied, the fraction examined and references concerning the analytical method.

TABLE 1

Outline of useful tests in quality studies of ice stored shrimp

Name of test	Type of extraction or separation	Pertinent reference
I. *Tests that indicate changes in prime quality*		
Glycogen-sugar content	5% Trichloroacetic acid containing 0.1% silver sulfate extract.	(16)
Lactic acid	5% Trichloroacetic acid extract.	(29)
Acid-soluble Orthophosphate	10% Trichloroacetic acid extract.	(29)
II. *Tests that signify the onset of spoilage*		
Trimethylamine nitrogen	5% Trichloroacetic acid extract.	(9)
Volatile acids	Steam distillation.	(13)
Bacterial plate counts	Aqueous extract.	(14)
Sulfhydryl groups	Aqueous extract. Myosin extract [isolated by method of Snow *(26)*].	(19)
III. *Other applicable tests for determining relative quality*		
Hydrogen ion concentration	Tissue homogenated in water.	
Amino nitrogen[1]	Aqueous extract.	(22)
Hydration of water-insoluble protein	Tissue homogenated in water.	
Sulfhydryl groups (Ferricyanide reduction)	Aqueous extract.	(15)

[1] Although amino nitrogen changes characteristically during the three quality periods outlined above, the changes are so minor that the test cannot be included in Class I or II.

EXPERIMENTAL PROCEDURE

The procedures for procuring, handling, and storing shrimp were those outlined by Fieger, Bailey, and Novak *(11)*. Headless, shelled, shrimp tissue was used for all determinations with the exception of bacterial counts. The shrimp tissue was cut into small pieces and 25 to 50 g. were extracted in a Waring blender employing the solvents listed in Table 1. Headless shrimp with shell intact were used for making bacterial counts. Other methods were as follows.

Determination of degree of hydration of water insoluble protein. A definite quantity of shelled shrimp (0.1 g/ml. water) was homogenated in a Waring blender and 50 ml. of this homogenate quickly transferred to graduated centrifuge tubes, and centrifuged 5 minutes at 1800 r.p.m. in a No. II International centrifuge. After permitting the centrifuge to stop without braking, the volume of insoluble precipitate was read immediately. The water-soluble fraction was then poured from the centrifuge tube, the insoluble fraction drained for 2 minutes, and approximately 0.5 g. examined gravimetrically for water.

Determination of hydrogen ion concentration. The pH was determined by use of the glass electrode on the homogenate obtained by blending samples as described under the previous heading.

RESULTS AND DISCUSSION

Three chemical tests have been found that measure definite changes during the prime quality period; these are tests for glycogen-sugar content, lactic acid content and trichloroacetic acid-soluble orthophosphate content. Non-specific reducing methods using copper or iron were unsatisfactory in determining carbohydrate in shrimp extracts because of the large number of non-sugar reducing substances found in this type of tissue. However, a procedure based on the rapid blood glucose method of Mendel *(18)* and revised for glycogen determination by Kleij *(16)* gave reproducible results. Since total hexose and pentose after hydrolysis is determined by this method, no distinction can be made between the glycogen and free sugar involved, but definite changes are manifest when the method is used for the analysis of the glycogen-sugar content of ice stored shrimp. Results from one of the experiments showed that these materials rapidly increased during the first three days of ice storage, then slowly decreased until the 6th day, after which time it rapidly decreased to a minimal value (Figure 1).[a] These relatively high values from the 2nd to the 6th day, correlate closely with the sweet taste of shrimp described above.

Lactic acid increased for the first few days (Figure 1), then decreased for the duration of the storage period. The decrease is less rapid after disappearance of the prime quality period. Initial concentration of this material would depend primarily upon the amount of struggling by the shrimp before death, but values for lactic acid during the ice-storage of two different lots of shrimp were in close agreement. This sharp increase during the first few days of storage is indicative of rapid breakdown of carbohydrate or other substances. Collins *(7)* found a similar increase in the lactic acid content of press juice of cod during the first few days of storage but could not account for the increase from glycolytic breakdown alone. Other workers have also failed to obtain accurate balances between

[a] All points shown in Figures presented in this paper were obtained by the method of moving averages described by Blair *(5)*.

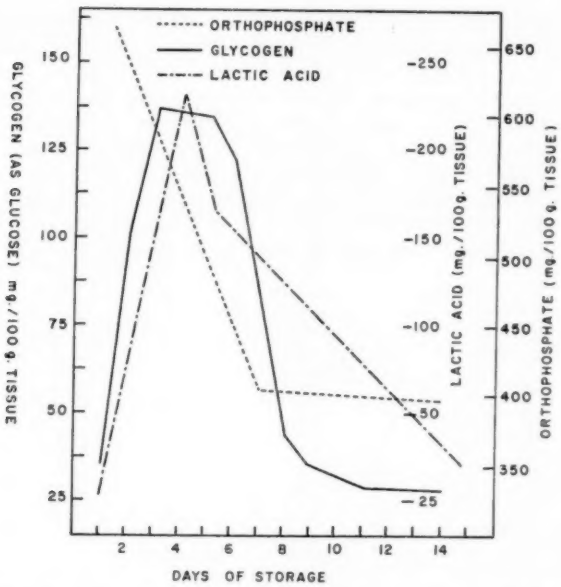

Figure 1. Effect of ice storage on orthophosphate, glycogen, and lactic acid content of shrimp.

glycogen decrease and lactic acid increase *(17, 26)*. After four days storage the lactic acid in shrimp was being oxidized faster than it was produced, resulting in a rapid decrease of this constituent. This was followed by a less rapid decrease for the remainder of the storage period.

Watson *(31)* postulated that lactic acid in the presence of trimethylamine oxide reacted to form carbon dioxide, water, and trimethylamine, and also demonstrated the disappearance of lactic acid and appearance of carbon dioxide and trimethylamine in a reducing *Achromobacter* suspension incubated with lactic acid and trimethylamine oxide. Collins *(7)* also found that both acetic acid and carbon dioxide increases concomitantly with decreases in lactic acid as further evidence for the operation of this reaction during the ice storage of fish. These changes are possibly applicable to shrimp deterioration, but other mechanisms are also operative since lactic acid does not decrease appreciably during the period of rapid rise in trimethylamine.

The acid soluble orthophosphate decreased very rapidly during the first few days of storage and then less rapidly throughout the remainder of the storage period (Figure 1). This change in the progression of decrease in orthophosphate occurs shortly after the shrimp have lost their prime quality. The mechanism for this decrease in acid soluble orthophosphate has not been elucidated, but preliminary experiments indicate a rapid leaching of inorganic phosphate from ice stored shrimp during the first few days of storage.

Most studies of protein denaturation indicate rapid release of free sulfhydryl groups during the process. Free sulfhydryl groups can be determined by different oxidizing reactions depending on the type and location of bonds broken as well as other physical characteristics as pH and temperature *(20)*. Since protein denaturation is involved in shrimp spoilage, definite changes should exist in sulfhydryl content during ice storage of this product. These changes occur in both the myosin and water-soluble fractions of shrimp protein, although varying results are obtained when different methods are used to determine this component (Figure 2). When iodine *(15)* is used as an oxidant, there is a decrease

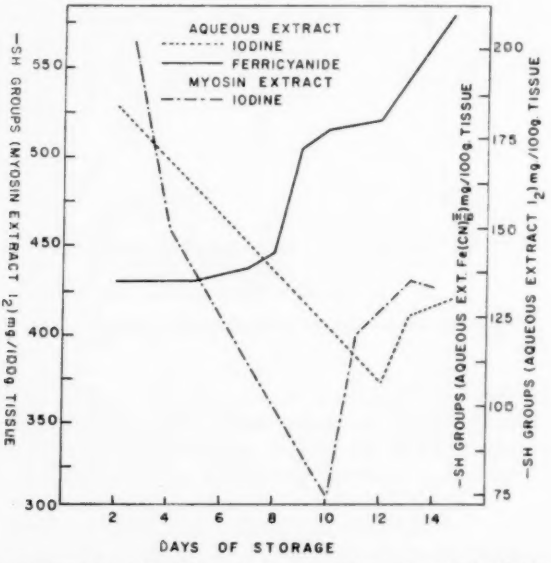

Figure 2. Changes in sulfhydryl content, as cysteine, of water-insoluble protein and of myosin during ice storage of shrimp.

in the titratable reducing groups in the water soluble and myosin fractions until spoilage progresses (Figure 2). Ferricyanide oxidizable groups of the aqueous extract increased continuously during ice storage of shrimp. Anson *(2, 3)* commented on the differences obtained in determining oxidizable groups by using the iodine and ferricyanide methods and elaborated upon the specific characteristics of these reactions. He showed that native egg albumin reacts with iodine but does not react with ferricyanide. He believes that the sulfhydryl groups present in native egg albumin are relatively inactive, but are accessible to iodine. Accessibility, he believes, is dependent upon charge of the individual reagents used as oxidants; ferricyanide being less accessible because of its negative charge. The initially high sulfhydryl groups found in fresh shrimp by iodine oxidation and the low values found by ferricyanide titration agree with this interpretation. Rapid denaturation occurring during spoilage apparently

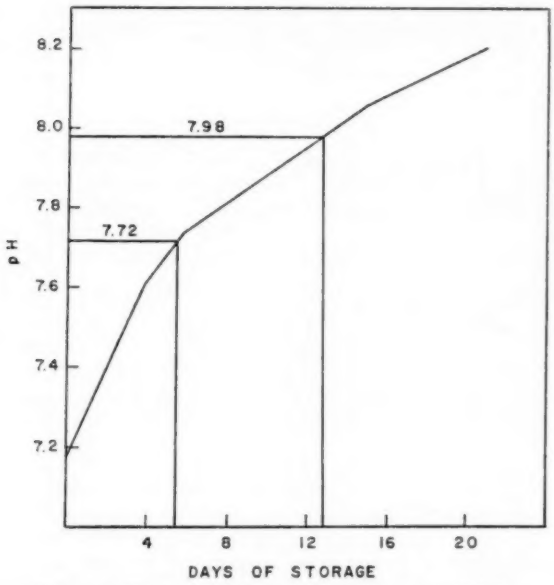

Figure 3. Changes in pH during ice storage of shrimp.

changes the structure of the protein near the sulfhydryl groups resulting in higher availability of these groups to both ferrieyanide and iodine.

The pH usually increases progressively from 7.2 to slightly over 8.2 after 21 days of storage (Figure 3). The curve shown in this graph represents a three period moving average *(5)* of the pH values obtained from shrimp samples of four different sizes taken from four different areas. If these data are representative of the pH of shrimp taken from other areas, a value of 7.7 or below is indicative of prime quality shrimp; those having values from 7.7 to 7.95 as poor quality but acceptable, and those having a pH of 7.95 or above as spoiled. These values are necessarily tentative in view of the above discussion.

Since free sulfhydryl groups tend to increase appreciably during the later phase of ice storage, there should also be an increase in the degree of hydration of water-insoluble protein due to association of water with sulfhydryl and other freed groups. This assumption was found to be valid by determining the degree of hydration of water-insoluble protein both by volumetric and gravimetric procedures. Representative results are presented in Figure 4. These results show that the volume of precipitate obtained is roughly proportional to the moisture content of the insoluble protein. This determination has been very useful in relative quality comparisons of shrimp stored in different types of ices when such ices do not affect the salt content of the shrimp. Salt content as high as 2 to 5% causes myosin swelling and greater quantities of salt cause rapid loss of water from the myosin fraction *(8)*. Thus, changes in salt content would necessarily invalidate the test.

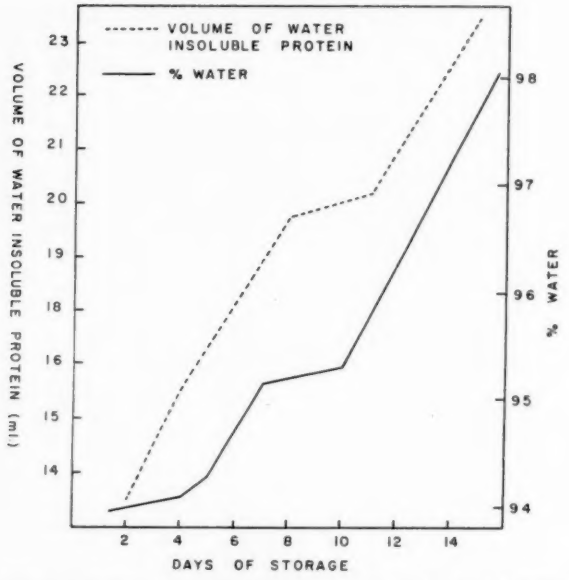

Figure 4. Changes in water content and volume of water-insoluble protein during ice storage of shrimp.

Other tests presently under investigation in this laboratory involve the determination of several B-complex vitamins and amino acids. Preliminary work shows that some of these constituents decrease in concentration during storage and these changes may be of value in determining the relative quality of shrimp and other seafoods.

SUMMARY AND CONCLUSIONS

A general review of chemical, physical and microbiological tests useful for determining the relative quality of ice stored shrimp is given. These tests are compared as indices of quality in relation to organoleptic changes in ice stored shrimp. The glycogen-sugar and the lactic acid and acid-soluble orthophosphate contents can be used for relative comparison of shrimp during their prime quality phase. Other useful tests are: pH, amino nitrogen, degree of hydration of water-insoluble protein, and B-vitamin content. Tests useful for determining onset of spoilage of ice stored shrimp are: trimethylamine nitrogen, volatile acids, bacterial content, sulfhydryl groups determined by iodine titration, and Nessler ammonia.

In most instances, individual tests are not conclusive for objectively qualifying shrimp, but rather are useful as relative quality indices when used in various combinations. However, a trimethylamine nitrogen value of 1.5 mg./100 g. shrimp tissue and a bacterial count of 10×10^6 per gram of headless, shell-on shrimp or higher are indicative of unacceptable shrimp in most instances. Tentatively, shrimp having a pH value below 7.7 can

be considered as prime quality, those having pH values between 7.7 and 7.95 as poor but acceptable and those having a pH value above 7.95 either as spoiled or on the borderline of spoilage.

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THE ABSORBABILITY OF NATURAL AND MODIFIED FATS*

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Most natural fats have been shown to be readily digested, but in the case of naturally-occurring fats of high melting point and of fats hardened by hydrogenation, the coefficient of digestibility may be reduced. Two major theories have been propounded to explain differences in digestibility: (1) that digestibility is inversely proportional to and dependent upon melting point *(3, 9)*; or (2) that digestibility is limited by the amount of saturated fatty acids of carbon chains 18 or longer *(8, 10)*. Other recent findings deserve consideration: Cheng, Morehouse, and Deuel *(2)* reported that monostearin was more digestible than either the triglyceride or the free fatty acid and Mattson, Baur, and Beck *(12)*, that long-chain saturated fatty acids were better utilized as diacetin fat than as triglyceride. These results indicate that not only is the chain length of the fatty acids imporant but also their association within the glyceride structure.

To provide basic information for the development of fats which will meet the Armed Forces' stability requirement of 160° F. these various factors were explored. Preliminary studies of lipid materials formed by interesterification with hexahydric alcohols were also conducted.

METHODS AND MATERIALS

Diets varying only in source of fat were fed to adult male albino rats, 5 to 7 per group, for 15 days and feces collected on the last 5 days for digestibility measurement. In each test a reference cottonseed-oil diet and a fat-free diet were fed. The amount of food offered was constant for all groups in each test and sufficient to permit small weight gains with the reference diet; in most cases consumption was complete.

The diets were composed of 80 parts of a diet base, as shown in Table 1, and 20 parts of test fat. Fats were dissolved in warm ethanol and mixed with the diet base; the diets were then placed on shallow trays and held at room temperature until the alcohol evaporated. This procedure was followed to insure intimate mixing of fat with other diet components. The fat-free diet was treated in the same manner with 80 parts of base added to 30.7 parts of sucrose to yield a diet iso-nitrogenous when equal calories were fed.

Fat content of the diet and of the feces was determined by a modified Association of Official Agricultural Chemists *(1)* procedure as follows:

> To approximately 2 g. of wet feces was added 5 ml. of concentrated hydrochloric acid and digestion carried out at low boiling temperature, with

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* This paper reports research undertaken at the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned No. 624 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

These findings were presented, in part, before the American Institute of Nutrition, 1956.

TABLE 1

Composition of diet base for fat test diets

Component	%
Casein	18.26
Methionine	0.49
Corn starch	37.50
Sucrose	36.25
USP Salts #2	5.00
Vitamin mixture[1]	1.25
Glycerine	1.25

[1] Supplying in milligrams per 100 g. of diet: Thiamine hydrochloride 0.50; riboflavin 0.50; pyridoxine hydrochloride 0.25; nicotinamide 2.00; calcium pantothenate 2.00; inositol 10.00; para-amino benzoic acid 5.00; biotin 0.01; folic acid 0.10; choline chloride 100.00; vitamin K 0.10; and vitamin B_{12} 50 μg. Fat-soluble vitamins were given by dropper prior to the collection period and provided 6000 I.U. of vitamin A, 60 I.U. of vitamin D and 5.0 mg. of alpha-tocopherol.

stirring, until all particles disintegrated. The digest was transferred to a Mojonnier flask, the beaker rinsed with a small volume of ethanol, and the mixture cooled. Lipids were extracted by adding 25 ml. of ethyl ether, shaking vigorously, adding 25 ml. of petroleum ether and shaking again. The extraction procedure was repeated three or more times and the ether phases transferred to a weighed crystallizing dish after each extraction. Evaporation was begun immediately in a fume hood and the evaporated sample dried to constant weight at 60-65° C. *in vacuo*. Recovery of 98.5 to 100% was obtained by this method from synthetic mixtures of free fatty acid, soap, neutral fat, and cholesterol. Absorbability was calculated from the formula:

$$\% \text{ Digestibility} = \frac{\text{Fat Intake} - (\text{Fecal Fat} - \text{Fecal Fat of Fat-free Group})}{\text{Fat Intake}} \times 100$$

The term "digestibility" is employed because of its general acceptance although it is recognized that the fecal lipids, particularly when fully hydrogenated fats are fed, contain appreciable amounts of soaps and some free fatty acids which represent end-products of digestion not absorbed. What is measured is a combination of both undigested and unabsorbed materials.

Data were analyzed statistically by the multiple range test of Duncan *(4)* for the significance of differences among ranked means or by the t-test *(5)* for the significance of the difference between two means.

Capillary melting point, iodine number, and saponification value of the fats were determined *(1)*. Seven natural fats—corn, soybean, cottonseed, coconut and palm oils, butterfat and lard—representing wide variation in melting point, saturation, and chain length were studied. Fully hydrogenated fats were prepared from these same materials. Characteristics of the natural and hydrogenated products are presented in Table 2.

Characteristics of the modified lards tested are listed in Table 3. The modifications were (1) butyration to the extent of one fatty acid equivalent, (2) interesterification with glycerol to form monoglycerides, and (3) interesterification with mannitol to replace glycerol, forming mixed mannitol esters. A simple mixture of hydrogenated lard and tributyrin compounded to yield approximately the same over-all fatty acid content served as a control for the butyrated lard. Hydrogenated lard monoglyceride was compared with glyceryl monostearate of higher melting point; and the mannitol esters with the normal glycerides from which they were made.

RESULTS AND DISCUSSION

Natural fats were found to be 97.0 to 99.7% digestible and these coefficients bore no relationship to melting point, saturation or chain length of the constituent fatty acids. The values obtained are given in Table 4,

TABLE 2

Characteristics of natural and hydrogenated fats

Fat	Capillary melting point ° C.	Iodine number	Saponification value	Saturated fatty acids C18 and above[1] % by weight
		Natural fats		
Butterfat	35.4	31	224	12.1
Coconut oil	25.4	10	237	2.7
Corn oil	< 0	125	192	4.2
Cottonseed oil	< 0	112	196	2.4
Lard	45.1	66	195	10.0
Palm oil	36.5	53	198	4.7
Soybean oil	< 0	126	193	3.3
		Hydrogenated fats		
Butterfat	49.8	< 1	224	33.8
Coconut oil	45.2	< 1	253	12.2
Corn oil	68.0	3	190	90.1
Cottonseed oil	61.6	1	195	72.3
Lard	61.2	< 1	195	73.4
Palm oil	59.4	1	198	57.3
Soybean oil	67.2	5	193	82.1

[1] Obtained from Hilditch (*7*).

TABLE 3

Characteristics of modified lards

Product	Capillary melting point ° C.	Iodine number	Saponification value
Butyrated hydrogenated lard (13.4% by weight)	52.9	< 1	236
Simple mixture of hydrogenated lard and tributyrin (81:19)	59.0	< 1	237
Monoglycerides of hydrogenated lard	67.6	13	160
Glyceryl monostearate	74.7	4	160
Mannitol esters of lard	30.7	63	192
Mannitol esters of hydrogenated lard	53.1	< 1	176

together with the statistical comparison of differences between means. Groups of means enclosed within a bracket do not differ from one another. In general, digestibility of the natural fats could not be clearly differentiated.

A similar comparison of the fully hydrogenated fats is shown in Table 4. Hydrogenated coconut oil was 98.5% digestible, significantly different from any other fat. Hydrogenated butterfat was superior to all fats except hydrogenated coconut, being 61.0% digestible. At the 1% significance level, hydrogenated corn oil differed from hydrogenated palm and hydrogenated cottonseed oils, but not from hydrogenated soybean oil or hydrogenated lard.

Representative body weight data are presented graphically in Figure 1. The highly digestible fats permitted adequate gains in body weight after adjustment to the change from stock diet. When a poorly digested fat was fed, the decreased caloric yield was reflected by lack of growth or small losses of body weight. Major losses of weight, indicative of toxicity of the test materials, were not encountered.

TABLE 4

Digestibility of natural and hydrogenated fats

Fat	% Digestibility	
Natural fats		
Coconut oil	.01[1]	.05[1]
Soybean oil	99.7 ± 0.07[2]	
Corn oil	99.0 ± 0.13	
Lard	98.6 ± 0.12	
Cottonseed oil	98.0 ± 0.16	
Butterfat	97.9 ± 0.02	
Palm oil	97.8 ± 0.62	
	97.0 ± 0.27	
Hydrogenated fats		
Coconut oil	.01	.05
Butterfat	98.5 ± 0.42	
Palm oil	61.0 ± 2.17	
Cottonseed oil	23.8 ± 3.04	
Soybean oil	22.8 ± 2.37	
Lard	17.2 ± 0.96	
Corn oil	15.2 ± 1.58	
	12.0 ± 3.08	

[1] Means enclosed within brackets do not differ at the indicated confidence level.
[2] Standard error.

A plot of the percentage digestibility of hydrogenated fats against melting point, illustrated in Figure 2, showed an inverse linear relationship with a correlation coefficient of –0.941. It should be noted, however, that the slope and definition of the line rely on the extreme values and that the data scatter in the very high melting point range. A better correlation ($r = +0.999$) was obtained when digestibility was plotted against saponification value, as in Figure 3. Digestibility varied directly with saponification value, or inversely, then, with the chain length of the fatty acids. Digestibility varied in curvilinear fashion with the calculated

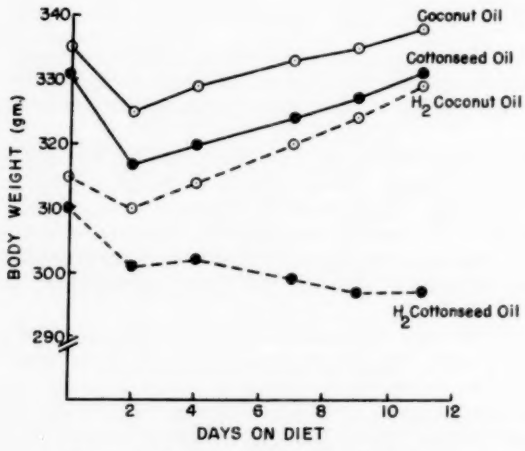

Figure 1. Representative body weight data.

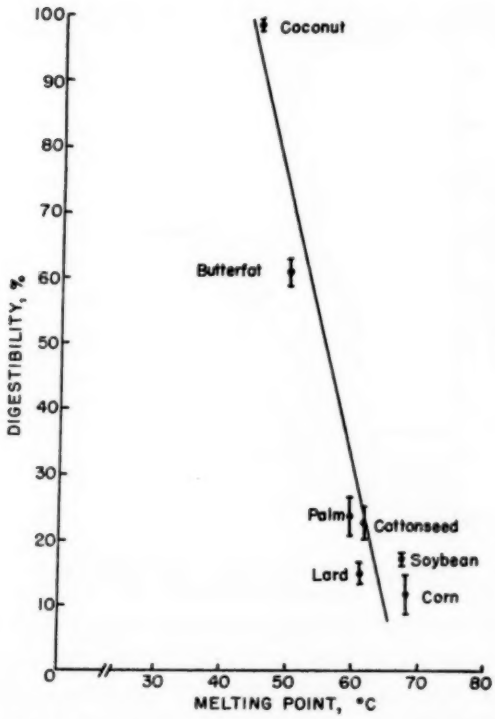

Figure 2. Relationship between melting point and digestibility of hydrogenated fats.

stearic acid content of the hydrogenated fats, the slope of the line changing at about 55% stearic acid. (See Figure 4). Extension of the curve to 100% stearic acid content resulted in a predicted value of about 11% digestibility for tristearin which agrees well with the reported value of 10.6% *(2)*. These various relationships suggest that the digestibility of stearic acid is not a constant and that its digestibility is a function of the other fatty acids contained in the glyceride structure. They also indicate that the relationship of digestibility and of melting point is coincidental, rather than causal, as melting point is determined by the component fatty acids of the fat. Verification was obtained in the studies of modified lards.

Butyrated hydrogenated lard was 47.7% digestible and the mixture of hydrogenated lard and tributyrin, 34.3%—a definite improvement over the 13.9% of the starting material. (See Table 5.) When digestibility of the hydrogenated lard fractions was calculated, assuming the butyryl groups to be 100% absorbable, it became apparent that mixing with tributyrin did not significantly improve absorption of the lard fatty acids—18.3% as compared with 13.9%—whereas butyration was distinctly beneficial, resulting in 35.4% absorption. Comparable results were obtained by Mattil and Higgins *(11)*, who concluded that the "solvent" action of oleic acid

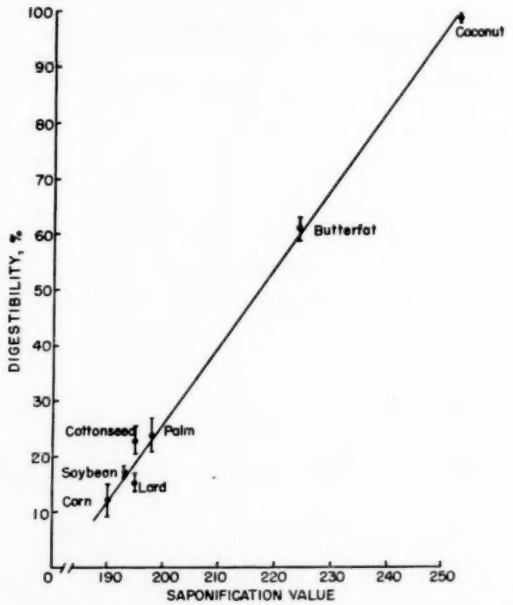

Figure 3. Relationship between saponification value and digestibilty of hydrogenated fats.

for stearic acid was markedly greater with mixed glycerides than with mixtures of triglycerides.

The monoglycerides of hydrogenated lard exhibited the same digestibility, 33.9%, as the lard fraction of the butyrated product. As shown in Table 6, stearic acid was equally as well absorbed from glyceryl monostearate, although its melting point was 7° C. higher than that of the lard monoglycerides.

Experimental results indicate that there is a maximum absorption value for stearic acid. This limit appears to be 30 to 40%.

The digestibility of glyceride lard was 98.6% and that of its mannitol esters, 95.5%. (See Table 7). This difference is statistically, if not practically, significant. No difference was noted between hydrogenated lard and the mannitol esters of hydrogenated lard, with digestibility values of

TABLE 5

Digestibility of butyrated lard

Fat	% Digestibility	
	Total	Lard fraction
Butyrated hydrogenate lard	47.7 ± 3.9[1] [2]	35.4 ± 4.8
Hydrogenated lard + tributyrin	34.3 ± 4.6	18.3 ± 3.9 [2]
Hydrogenated lard	13.9 ± 1.2	13.9 ± 1.2

[1] Standard error.
[2] Means within brackets do not differ at the 5% level of significance.

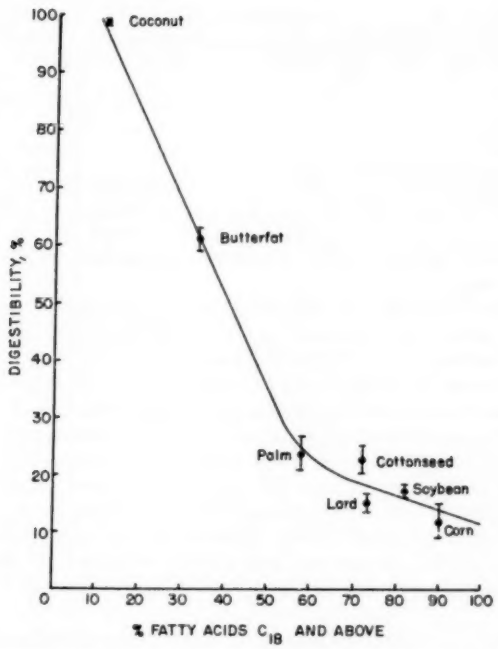

Figure 4. Relationship between content of long-chain fatty acids and digestibility of hydrogenated fats.

19.3% and 18.9%, respectively. The esters of mannitol are seemingly as susceptible to lipolysis as those of glycerol and their absorption a function of the fatty acid composition as in the glycerides. Mannitol esters of olive oil, a by-product of glycerine production, were studied by Halliburton, Drummond, and Cannon *(6)* and found to be utilized to practically the same extent as olive oil, 95.8% as opposed to 96.6%. The small reductions in digestibility which have been noted may be due to poorer absorption of the mannitol than of glycerol.

SUMMARY AND CONCLUSIONS

Mature rats were fed a series of natural and modified fats in order to determine the relationships among melting point, saturation, chain length, structure and digestibility. Twenty per cent fat was incorporated into a

TABLE 6

Digestibility of monoglycerides

Fat	% Digestibility
Monoglycerides of hydrogenated lard	33.9 ± 3.0 [1] [2]
Glyceryl monostearate	31.8 ± 3.9 [2]
Hydrogenated lard	13.9 ± 1.2

[1] Standard error.
[2] Means within brackets do not differ at the 5% level of significance.

TABLE 7

Digestibility of mannitol-lard esters

Fat	% Digestibility
Lard	98.6 ± 0.21 [1]
Mannitol-lard esters	95.5 ± 0.34
Hydrogenated lard	19.3 ± 1.29] [2]
Mannitol-hydrogenated lard esters	18.9 ± 2.04]

[1] Standard error.
[2] Means within brackets do not differ at the 5% significance level.

purified diet fed for two weeks. Feces were collected during the last five days for fat analysis.

Natural fats included in the study were: cottonseed, soybean, corn, coconut and palm oils, lard and butterfat. Digestibility was not related to the characteristics cited. When these same fats were fully hydrogenated, digestibility varied inversely in linear fashion with the chain length of the constituent fatty acids and in curvilinear fashion with the amount of saturated acids C_{18} and above. The general inverse linear relation between digestibility and melting point is ascribed to the relationship which exists between the melting point and the amounts of component fatty acids.

The monoglyceride of hydrogenated lard was found to be more digestible than the original triglyceride. Substitution of one-third of the fatty acid radicals by butyryl groups was equally effective in raising digestibility; while simple mixture of tributyrin with hydrogenated lard showed no effect.

Digestibility of mannitol esters of lards was similar to that of the glyceride lards from which they were made, indicating that digestibility was a function of the constituent fatty acids.

It is concluded that digestibility is primarily dependent upon the amounts and chain length of the saturated fatty acids and their arrangement within the glyceride structure.

Acknowledgments

The authors are indebted to Dr. Robert Vander Wal of Armour and Company for supplying the butyrated and hydrogenated lards; and to Drs. Harry Spector and Herbert Hollender of the Quartermaster Food and Container Institute for the Armed Forces for their interest in the conduct of these studies and review of the manuscript.

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FREEZE-DRIED MEAT. III. NON-OXIDATIVE DETERIORATION OF FREEZE-DRIED BEEF[a]

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Freeze-dried meat has been shown to be a desirable product with respect to versatility and acceptability *(18)*.

Another necessary requirement for a dehydrated food is good storage stability at relatively high temperatures. The deterioration of freeze-dried beef can be quite extensive under adverse conditions or extended storage. The mechanism of oxidative deterioration has been the subject of previous research *(17)*. Since the oxidative deterioration of the freeze-dried meat must be eliminated by packing under vacuum or inert gas, in practice the non-oxidative deteriorative reactions become the most important factor in storage.

In his review on dehydrated meat Sharp *(14)* has indicated that oxidation can be extensive. However, it becomes insignificant when the dehydrated meat is compressed or packed under nitrogen. Under these conditions the non-oxidative reactions predominate. Sharp *(15)* has recently provided evidence that this deterioration mechanism is active-carbonyl amine browning with glucose and phosphorylated sugars as the most important reactants. The recent work of Hendrickson *et al. (3, 4)* on precooked dehydrated pork has shown that the browning reaction is probably an important factor in the storage deterioration, for the oxidation of glucose by glucose oxidase has a highly beneficial effect on the storage stability. This approach is apparently a valid analogy to similar studies of the storage deterioration of dehydrated eggs. The removal of glucose from eggs by fermentation by yeast or oxidation by glucose oxidase has been effectively used to decrease the deterioration of this product *(10)*. The review by Coulter, Jenness, and Geddes *(2)* points up the importance of the browning reaction in the storage of dry milk. It therefore appears that the browning reaction could be one of the major, if not the most important non-oxidative deterioration mechanism in freeze-dried meat.

It is the purpose of this research to define the major physical and chemical changes which occur during the storage deterioration of freeze-dried beef. On the basis of the changes found possible reaction mechanisms are analyzed.

EXPERIMENTAL

Preparation of material. Pieces of *biceps femoris* of beef of about one-inch thickness were freeze-dried at a plate temperature of 40° C. and pressure of 0.1 mm. in a

[a] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

Stokes model 2004L freeze-drier *(18)*. In all the studies except for those of fluorescence and color, the dry beef was then ground in a Wiley mill to about No. 10 mesh. Ground dry beef was hydrated to desired water content and was then placed in screw top jars. Jars were placed in a vacuum chamber, evacuated and filled with nitrogen three times before sealing. The beef was placed in storage at the desired temperature. At the end of the storage period, the material was transferred to a −18° C. cold storage room until analyses.

Reducing compounds. The reducing compounds in freeze-dried beef were determined by ferricyanide reduction using the method of Chapman and McFarlane *(1)*.

Fluorescence. Fluorescent components of stored freeze-dried beef were found to be insoluble in any simple solvent. The following direct method was devised to measure the changes in the fluorescence during storage. A 2 x 37 x 11 mm. slice of the dry beef was inserted in the cuvette holder of a Beckman DU spectrophotometer and its fluorescence was compared with a 0.2 mg./l. solution of quinine sulfate used as a standard of 100. The slices of meat were taken off the same piece of meat throughout the experiment to insure similarity of texture and grain. The slice of meat was carefully fitted into the cuvette holder at an angle such that its upper end was closest to the photocell compartment. Thus the ultra-violet light entering the bottom of the cuvette holder struck the slanted surface of the slice and the emitted light (fluorescence), which was mostly in the visible range, was measured with the photocell. Any ultra-violet light that was reflected was absorbed by the filter between the sample and the photocell.

Amino nitrogen. The Van Slyke manometric method for free amino groups was found to be inapplicable because the beef precipitated on the walls of the reaction vessel and therefore would not stay in contact with the nitrous acid solution. The method used was a formol titration with modifications suggested by Shipstead *(16)*. One g. of freeze-dried ground beef was placed in the cup of a Waring blender with 250 ml. of 11 g./100 ml. sodium salicylate solution as a peptizing agent and two drops of silicone antifoam. The mixture was blended at reduced speed for 30 minutes. The solution was then transferred to a 600 ml. beaker using 50 ml. of salicylate solution to complete the transfer. The solution was titrated with 0.1N. NaOH to pH 9.0 on the glass electrode meter. Ten ml. of reagent grade formaldehyde were added and the solution retitrated to pH 9.0. Five min. were allowed to complete the titration in both cases.

Soluble nitrogen. Two g. of ground dry beef and 2 g. of powdered glass were placed in a mortar. Enough 0.5 N.KCl was added to thoroughly wet the material. The mixture was ground, transferred to a 200 ml. volumetric flask with 0.5 N. KCl and made to volume with this solution. The extraction was allowed to proceed with occasional shaking. At the end of 2 hours, the solution was centrifuged at 2000 times gravity for 15 min. The total soluble nitrogen was determined on 50 ml. of the supernatant by the Kjeldahl method *(6)*.

Soluble hematin compounds. The soluble hematin pigments in the supernatant from the KCl extraction for soluble nitrogen were determined by their spectral absorbance at 412 mμ in a Beckman DU spectrophotometer.

Soluble proteins. The non-protein nitrogen was also determined on the KCl extract supernatant. Ten ml. of 30 g./100 ml. trichloroacetic acid were added to 50 ml. of the supernatant from the soluble nitrogen preparation. After mixing well and letting stand for 15 min., the solution was centrifuged at 2000 times gravity for 15 min. The soluble non-protein nitrogen was determined on 50 ml. of this supernatant by the Kjeldahl method. The soluble protein was calculated by difference.

Enzyme digestibility. Five g. of ground freeze-dried beef were rehydrated with 30 ml. of distilled water at 23° C. for 30 min. One g. of papain (Nutritional Biochemicals) was wetted and dispersed in a solution containing 50 ml. water, 5 ml. 0.03 M. Versene and 1 ml. 0.15 M. cysteine as activators. This solution was diluted to 100 ml. and filtered. Five ml. of the papain solution were added to the beef suspension. The digestion was allowed to proceed at 23° C. for 2 hours with occasional stirring. The suspension was transferred to a 200 ml. volumetric flask, diluted to about 140 ml. with water and 50 ml. of 30 g./100 ml. trichloroacetic acid were added to stop the reaction and precipitate the protein. After dilution to volume and mixing, the suspension stood

15 min. before centrifuging at 2000 times gravity for 15 min. The amino acids in the supernatant were determined by the colorimetric method of Troll and Cannan *(19)*.

Protein nutritive value. The nutritive value of the freeze-dried beef was determined with a bioassay technique utilizing 10-day-old male chicks. To a basic mixture of essential dietary components, ground dry beef was added to provide 20% crude protein in the final diet. Each sample was fed to 8 birds chosen at random from an optimum growth group. The average per cent weight gain per day for an eight-day period is taken as the indicator of the nutritional value.

Sugars. Twenty g. of dehydrated beef were extracted with 80% ethanol in a soxhlet apparatus for 4 hours. After removal of the ethanol on a steam bath, the volume was brought to 60 ml. with water. The fat was extracted with two 200 ml. portions of chloroform. The chloroform-water emulsion was broken by centrifuging at 1000 times gravity. The yellow water phase was passed over anionic and cationic exchange resins to remove the salts which interfere with chromatography on paper. The final solution was concentrated to 2 ml. and 15 μl. were placed on a spot on 18 x 21 in. Whatman No. 1 filter paper. The chromatograms were run in 80% phenol in water (23 hr. for 18 in. distance) and 52.5% n-butanol–32% ethanol–15.5% water (16 hr. for 21 in. distance). After drying, the papers were sprayed with m-phenylenediamine. When the spray had dried, the papers were heated 3 to 4 min. at 100° C. to develop the color. Standard chromatograms were prepared using known sugars.

Rehydration. Weighed pieces of freeze-dried beef approximately $1 \times 1 \times 2$ inches were placed in aluminum foil boats with cross sections about the size of the pieces. A quantity of water equal to 3 times the weight of the meat was added to each piece. Weights were placed on top of the pieces of meat to prevent their floating during the rehydration. After rehydration for 2 hrs., the pieces of meat were drained and the excess water blotted off. The rehydrated meat was weighed and the rehydration ratios calculated.

Color. Observation of color changes of freeze-dried meat during storage were made visually. Also, the color change was measured on a Colormaster Differential Colorimeter (Manufacturers Engineering and Equipment Corp., Hatboro, Pennsylvania). For the instrumental determination of color, pieces of freeze-dried beef $1 \times 2 \times 2$ in. were placed in pint mason jars which had their bottoms replaced by flat cover glasses of known reflectance. The jars were placed in a vacuum chamber, evacuated and filled with nitrogen gas. After closing the lids of the jars were sealed with sealing wax. The material was placed in storage and the jars were removed periodically for measurement of the color.

RESULTS

Reducing compounds. Figure 1 shows the increase in reducing compounds as measured by the ferricyanide reduction of freeze-dried beef containing 2.4% water stored at 35° C. Glucose does not cause reduction of ferricyanide under the experimental conditions. Thus the increase must be due to sulfhydryl groups and other readily oxidized compounds which are produced by the deteriorative reactions.

Fluorescence. Increase in fluorescence with time of storage at 35° C. is shown in Figure 2. The fluorescence apparently reached a maximum at about 35 days.

Free amino groups. A decrease in the free amino groups was found during storage at 54.4° C. (Figure 3) for a sample containing 3% water. The initial value was 0.05 g. free amino nitrogen per g. total nitrogen.

Soluble nitrogen. The middle curve of Figure 4 indicates the large decrease in KCl soluble nitrogen. The meat used in this experiment contained 3% water. The initial solubility was 22.35 mg.N./50 ml. KCl solution or 44.7 mg.N./g. dry meat. This is equivalent to about 37% of the

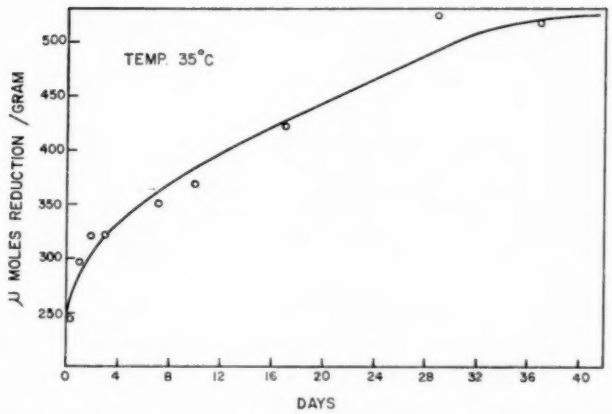

Figure 1. Reducing compounds formed during storage of freeze-dried beef.

total nitrogen, a value similar to that observed by Wierbicki *et al. (21)* with fresh meat.

Soluble protein. Curves 1 and 3 in Figure 4 show the decrease in KCl soluble non-protein nitrogen and protein nitrogen. The values are in per cent of the initial which were 8.00 and 14.35 mg. N./50 ml. KCl solution, respectively. The initial soluble protein is equivalent to about 18% of the weight of the dry beef.

Hematin pigments. The absorbance at 412 mμ of the KCl extracts of meat stored at 54.4° C. found in Figure 5 are in per cent of the initial absorbance which was 1.25. This absorbance corresponds to a hematin concentration of about 1×10^{-5} M. The soluble nitrogen was 24.4 mg. nitrogen/50 ml. of KCl extract in the initial sample. The absorbance at

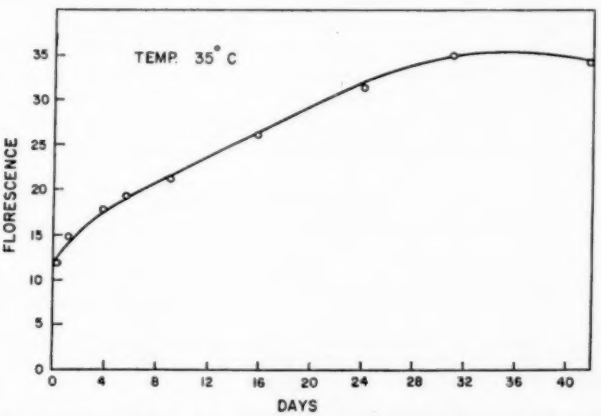

Figure 2. Increase in fluorescence during storage of freeze-dried beef.

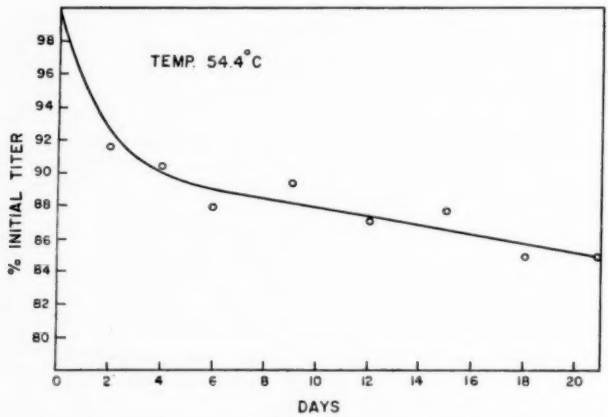

Figure 3. Decrease in free amino groups during storage of freeze-dried beef.

412 mμ is essentially a measure of the total hematin pigments for the molecular extinction coefficients for myoglobin, oxymyoglobin and metmyoglobin are nearly equal at this wave length *(8)*.

Enzyme digestibility. The digestibility of freeze-dried beef by papain after storage at 54.4° C. and 3% water is shown in Figure 5. The initial digestibility was 0.109 millimoles amino acid released from 1 g. of meat in 2 hours. Since some of the lower peptides are soluble in 7.5% trichloroacetic acid, these contribute to the color formed by reaction with ninhydrin although their reaction is not quantitative *(19)*.

Nutritive value. Average growth rates of chicks fed freeze-dried beef after it had been stored at 54.4° C. were 5.1% gain/day for the control,

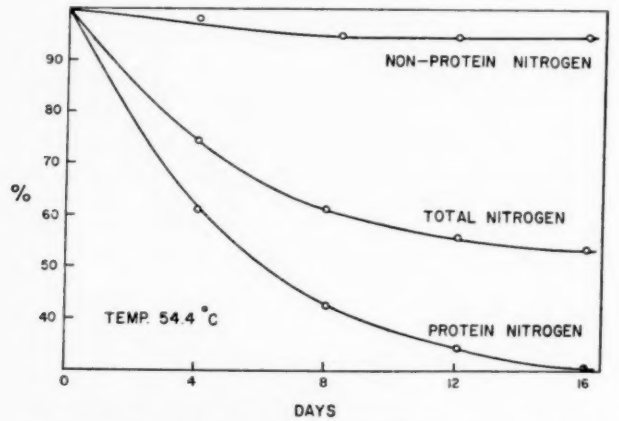

Figure 4. Decrease in soluble total, non-protein and protein nitrogen during storage of freeze-dried beef.

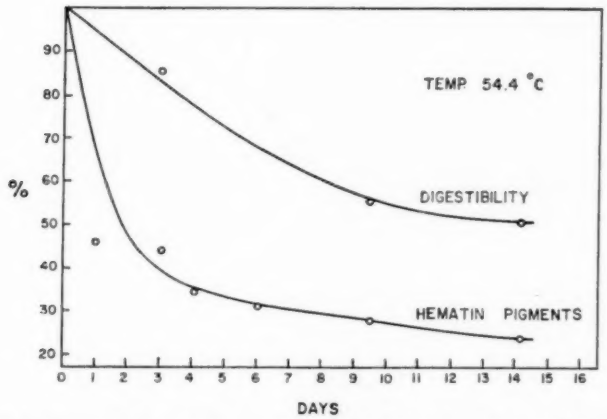

Figure 5. Decrease in soluble hematin pigments and papain digestibility during storage of freeze-dried beef.

5.6% gain/day for the sample stored 20 days, and 5.9% gain/day for the sample stored 30 days. Statistical analysis showed that there is no significant difference between these three growth rates. The material used in this study had a moisture content of 5.9% on the dry basis and 12.1% nitrogen. A rate of 5% gain/day is considered good but not excellent growth.

Sugars. Glucose, mannose, and fructose were the only sugars detected in the uncombined state. The glucose and fructose levels were apparently equal, while that of mannose was somewhat less in the freshly freeze-dried beef. A sample stored 16 days at 35° C. showed no change in fructose but a large decrease in glucose and mannose.

Rehydration. The rehydration capacity of freeze-dried beef decreases quite significantly during storage. Table 1 gives some limited results for storage at two temperatures. The material used in these studies contained 3% water on the dry basis. The rehydration ratios are the ratio of the weight of water taken up by the dry meat to that which would be necessary to make the rehydrated meat 75% water. Different batches of freeze-dried beef were used for storage at 35.5 and 54.5° C. The difference in the rehydration ratios for the control samples are mostly due to quite large

TABLE 1

The effect of storage on the rehydration of freeze-dried beef

Sample	Temperature C.	Time months	Rehydration ratio	% of control
A1	−17.8°	9	0.818	100
A2	35.5°	4	0.788	96.4
A3	35.5°	9	0.380	46.5
B1	−17.8°	4	0.508	100
B2	54.4°	2	0.285	56.2
B3	54.4°	4	0.168	33.1

differences in fat content of the beef. Comparisons should only be made within the batches.

Color. The color of freshly freeze-dried beef is a light pink. On storage at temperatures above freezing, the pink color is supplanted by a light grey-brown. When the deterioration is quite severe as shown by other analytical methods, the color progresses to brown, yellow-brown and then orange brown. If meat which has reached the yellow-brown stage is rehydrated and cooked, it has a dark brown burned appearance. The chromaticity curve for freeze-dried beef stored under nitrogen was calculated from the readings on the Colormaster Differential Colorimeter by the method indicated in the instruction manual and is shown in Figure 6. Initial

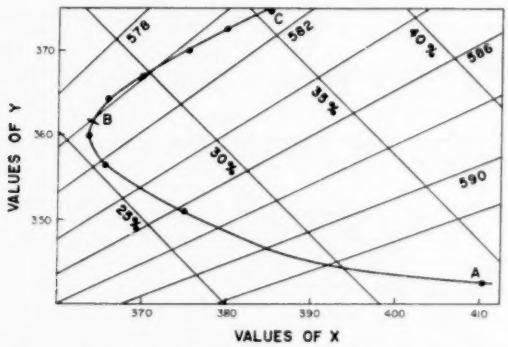

Figure 6. Color changes during storage of freeze-dried beef shown as a curve on a partial chromaticity diagram. This diagram includes the dominant wavelength region 578 mμ to 592 mμ and excitation purities of 25% to 40%.

readings on the sample, which contained about 3% water, correspond to point A on the curve. In 43 days at 35.5° C. the readings had progressed along the curve to point B. At this time, the sample was transferred to storage at 54.4° C. During 72 days at 54.4° C. the color had changed to correspond to point C. Initial storage at 35.5° C. caused a large change in the dominant wave length of the color together with a sizeable decrease in excitation purity. Subsequent storage at 54.4° C. caused a large increase in excitation purity with only small changes in the dominant wave length. The large decrease in dominant wave length corresponds to the loss of the pink color and the development of the brown metmyoglobin color. The final increase in excitation purity with the small change in dominant wave length is related to the development of a yellow-brown color.

DISCUSSION

The possible non-oxidative deteriorative reactions in freeze-dried beef during storage are rather limited. The moisture content is so low that mold growth may be eliminated from consideration *(11)*. Also, enzymatic reactions would be non-existent or at least negligible at this moisture level. Since oxygen has been excluded from the beef during these storage studies, the beef triglycerides and similar lipids will be stable.

Some compounds in the beef could undergo thermal decomposition. This has been shown to occur qualitatively since on long storage, the freeze-dried meat develops a putrid amine- or ammonia-like odor. However, none of the evidence points to any large amount of reaction by this type of mechanism.

The decrease in rehydratibility, protein and hematin pigment solubility and the increase in reducing compounds might seem to be indicative of thermal protein denaturation *(12)*. However, these changes are also characteristic of browning *(5, 7, 20)*.

The color changes, development of fluorescence, loss of free sugars and decrease in formol titratable amino groups are all known to occur in active carbonyl-amine browning *(5, 6)*.

Since the raw material used was fresh beef and freeze-drying is the mildest method known for drying materials, the proteins of meat should have undergone the minimal amount of heat denaturation during the preparation. Thus, much of the protein should be in the native state at the beginning of storage. If thermal denaturation did occur during storage an increase in digestibility by proteolytic enzymes would probably result *(13)*. However, the digestibility with papain was found to decrease adding further evidence against thermal protein denaturation.

Reactants for the browning reaction are certainly available in meat for there are present, in addition to the protein and amino acids, residual glucose, hexose phosphates, organic acids and other metabolic intermediates. It can therefore be concluded that the active carbonyl-amine browning reaction is the most important deteriorative mechanism in the storage deterioration of freeze-dried meat.

The results from the bioassay of the nutritional value of the stored freeze-dried beef show that the change in nutritional value is not a critical factor in its use. The changes in rehydratability, color and flavor would be so great as to make the meat organoleptically unacceptable before any significant change would have occurred in the nutritional value of the protein.

SUMMARY

The non-oxidative deterioration of freeze-dried beef was characterized by physical and chemical measurements made on samples stored in nitrogen atmospheres at controlled temperatures and water contents. The following changes were observed:

1. Reducing compounds increased.
2. Fluorescence increased.
3. Free amino groups decreased.
4. There was a decrease in soluble total, non-protein and protein nitrogen.
5. Hematin pigments became less soluble.
6. Papain digestibility decreased.
7. Free glucose and mannose decreased.
8. Rehydration capacity was lowered.
9. The color changed from light pink to brown to yellow-brown.

All of these changes are good criteria for active carbonyl-amine browning. It is concluded that active carbonyl-amine browning is the main and probably the only significant non-oxidative deteriorative reaction.

Even freeze-dried meat rendered organoleptically unacceptable by deterioration showed no loss in protein nutritive value.

Acknowledgments

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FREEZE-DRIED MEAT. IV. FACTORS AFFECTING THE RATE OF DETERIORATION[a]

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A large amount of evidence suggests that the major deteriorative reaction in freeze-dried beef is active carbonyl-amine browning *(15)*. Studies of active-carbonyl-amine browning in model systems *(4, 10, 13, 22, 23)* and in dehydrated food commodities *(3, 8, 11, 12, 16, 17, 18, 21)* have defined the major factors affecting the reaction rate of browning. These factors include temperature, water content, pH, concentration of carbonyl compounds, particularly glucose, and the presence of inhibitors.

Mohammad *et al. (13)* found an activation energy of 30 kcal. for glucose-bovine serum albumin browning. Lea and Hannan *(4)*, using freeze-dried glucose-casein, found that browning conformed to the Arrhenius equation over the temperature range 0 to 90° C. with a $Q_{10}{}^{15\text{-}25^\circ C.}$ of 5.4. The browning reaction in apricots *(17)* conformed to the Arrhenius equation with an activation energy of 26 kcal. Considering the high temperature coefficient of these examples of browning, it is important to define quantitatively the effect of temperature on browning in freeze-dried beef.

Lea and Hannan *(10)* found that the rate of browning of freeze-dried glucose-casein reached a maximum of 70% relative humidity at 37° C. Browning deterioration in dehydrated eggs *(12, 18)*, dehydrated milk *(3)* and dehydrated meat *(16)* greatly increases at high (5-10%) water contents. Sharp *(16)* summarizes the studies of the British researchers showing that dehydrated beef and pork and freeze-dried beef undergo greatest deterioration at the higher (7-9%) water contents. Because the cost of removing water from freeze-dried meats, especially below 10%, is one of the major economic problems, the importance of very low water contents for freeze-dried meats must be quantitatively evaluated. Thus, it is pertinent to quantitate the effect of water content on the browning deterioration of freeze-dried beef.

Fundamental studies of active carbonyl-amine browning have shown *(9, 10, 13)* that hydroxyl ions catalyze and hydrogen ions inhibit. In dehydrated eggs, lowering the pH to inhibit browning *(12)* was at one time commercially practiced. Perhaps an adjustment of the pH in freeze-dried beef could be used to inhibit its browning deterioration.

Removal of the reactant, glucose, is commercially practiced in the dehydrated egg industry. Henrickson *et al. (5, 6)* found that the lowering of the glucose concentration in dehydrated pork increases its storage stability.

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The removal of glucose, one of the main active carbonyl reactants in freeze-dried beef, should be given consideration.

Carbonyl reactants, especially sulfites, are good inhibitors of browning, and the use of sulfur dioxide as a browning inhibitor in the dehydrated fruit is standard practice. Specific inhibitors which react with carbonyl compounds help in defining the browning mechanism in freeze-dried meats, and could prove useful in extending the storage life.

This report describes the quantitative effect of temperature, water content, pH, concentration of carbonyl compounds and the presence of inhibitors on the browning deterioration of freeze-dried beef.

EXPERIMENTAL

Effect of temperature. For an investigation of the effect of temperature on the rate of deterioration, a large quantity of freeze-dried *biceps femoris* of beef was ground in a Wiley mill. It was then well mixed by hand, divided into a number of small samples, and bottled. The bottles were evacuated, filled with nitrogen, and closed. The moisture content was 3% as determined by the weight loss after drying in a vacuum desiccator containing solid magnesium perchlorate for 8 days at 20° C. *(20)*. The material was placed in storage at 21.1, 35.0, 54.4, and 71.1° C. Samples were removed from storage periodically and kept in a cold room at −18° C. until analyzed. These samples were analyzed for free amino groups, KCl soluble nitrogen and soluble hematin compounds by the methods described previously *(15)*.

Effect of additives. To determine the effect of various additives on the rate of freeze-dried beef deterioration, the following method for incorporation of these additives was used: A large quantity of freeze-dried beef was coarsely ground in a Wiley mill and then finely ground in a Holwich grinder. After mixing well, 160 g. were blended with one l. of water containing the desired additive for 2 min. for each sample. The slurry was then frozen in shallow pans and freeze-dried. The additives used were water; pyruvic acid, 0.38%; lactic acid, 0.5%; semicarbazide, 0.3%; sodium bisulfite, 0.3%; glucose, 0.3% and 0.5%, and glucose oxidase (Dee Gee), 0.25%. The amounts of the additives are in % of the weight of dry meat used. In the sample with glucose oxidase, 4 ml. of 30% hydrogen peroxide were added to the slurry which was allowed to stand 3 hr. at room temperature before freezing and drying. Since the meat was in the form of a non-friable porous cake after drying, it was reground to a powder in the Wiley mill. After bottling, the material was put in a vacuum chamber which had an open container of sulfuric acid at a concentration such that the relative humidity was 65%. The material was equilibrated under vacuum for 2 days. The vacuum was released with nitrogen, and the bottles capped. The samples were stored at 54.4° C. for varying periods of time before analysis.

Effect of pH. To determine the effect of pH on the rate of deterioration, samples were prepared in a manner similar to that above, except that the slurries were adjusted to the desired pH with HCl or KOH. The pH's used were 3.0, 4.5, 6.0, 7.5, and 9.0. The material containing the various additives as well as that of the several pH's was analyzed for KCl soluble protein by the method noted previously *(15)*.

Effect of volatile components. Cationic and anionic exchange resins and activated carbon were placed in separate vacuum chambers with samples of ground freeze-dried beef. A quantity of saturated magnesium chloride ($MgCl_2 \cdot 6H_2O$) in contact with excess solid was also put in the chamber to eliminate the possible variability in the activity of water. The chambers were stored at 35.5° C., and samples were removed periodically for analysis by the KCl soluble protein method.

Effect of moisture content. The moisture content is known to be very important in the deteriorative changes in dehydrated foods. A quantity of well ground freeze-dried beef was mixed and divided into a number of small samples in bottles. To obtain samples with different moisture contents, portions of the material were placed in vacuum chambers with sulfuric acid solutions of different concentrations (25, 35, 45, and 60 wt. %), and solid magnesium perchlorate as hygrostats. The material was

allowed to equilibrate at 21° C. for 8 days. The vacuum was released with nitrogen, and the bottles capped. A portion of the material from each chamber was set aside for moisture determination while the rest of it was placed in storage at 54.4° C. Samples were removed periodically and analyzed for KCL soluble protein. The moisture levels of the samples were determined by drying in a vacuum desiccator over magnesium perchlorate for 8 days at 20° C.

Accelerated browning with additives. The preparation of samples of ground freeze-dried beef with several intermediary metabolites, suspected browning intermediates, and antioxidants has been described previously *(19)*. The additives used were glucose, glycogen, lactic acid, fructose-1,6-diphosphate, ascorbic acid, morpholino-glucose reductone, piperidino-glucose reductone, dimethyl amino-glucose reductone, nordihydroguaiaretic acid (NDGA) and propyl gallate.

The samples were placed in small vials and sealed under nitrogen. Browning was measured at 100° C. by visual scoring referred to standards made up of mixtures of fresh freeze-dried and severely browned freeze-dried beef. The standards which were given scores of 0, 2, 4, 6, and 8 had Munsell color designations of 2.5 Y 7/4, 7.5 YR 6/6, 7.5 YR 5/6, 5 YR 5/8, and 2.5 YR 5/6, respectively. The sample vials were placed in an oven at 100° C. for 4.2 hours, after which time they were compared with the standards.

RESULTS

Temperature effect. The results of the formol titration of the free amino groups of the samples stored at several temperatures shown in Figure 1, are in per cent of the initial value. The initial formol titer was

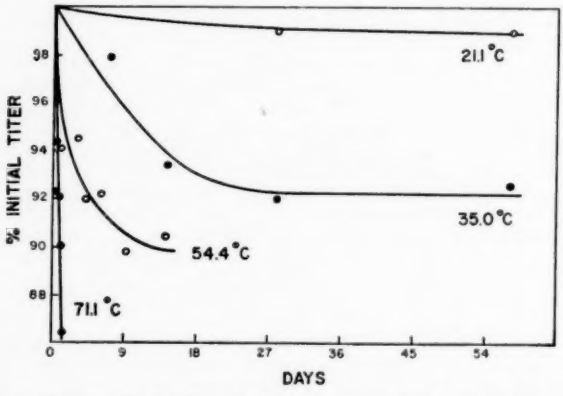

Figure 1. Decrease in the formol titer of freeze-dried beef during storage.

72.8 mg. amino nitrogen per g. of dry meat. The rates at the several temperatures were estimated from the time necessary for a decrease in 5% of the formol titer. The amount of decrease in the formol titer of the sample stored at 21.1° C. was too small for an accurate estimate of the rate.

The results of the analyses of the K Cl solution extracts of the samples stored at the several temperatures are given in Figures 2 (soluble nitrogen) and 3 (absorbance at 412 mμ). The values are in per cent of the initial values which were 24.4 mg. soluble nitrogen per 50 ml. of K Cl extract, and an absorbance of 1.29 at 412 mμ. The rates here were estimated from the time necessary for a 15% decrease in soluble nitrogen and a 50% decrease

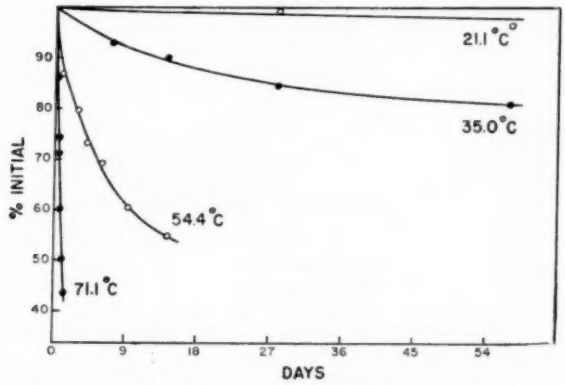

Figure 2. Decrease in the solubility of nitrogenous compounds in freeze-dried beef during storage.

in the absorbance at 412 mμ. The Arrhenius plots of these estimated rates are given in Figure 4. The apparent activation energies were calculated from the slopes of the curves using the integrated form of the Arrhenius equation. The values for the apparent activation energies are 26 kcal/mole for the formol titer, 27.4 for the K Cl soluble nitrogen and 24 for the absorbance at 412 mμ.

Effect of additive. The decreases in K Cl soluble protein for the samples with various additives are plotted in Figure 5. The results for certain pairs of samples were so similar that the eight samples can be represented by four curves. The initial values for these samples in mg. protein nitrogen/50 ml. K Cl extract were: control, 14.35; pyruvate, 13.15; lactate, 13.55; semicarbazide, 12.90; bisulfite, 14.62; 0.3% glucose, 13.62; 0.5% glucose, 14.04, and glucose oxidase, 10.93. The initial value for the glucose

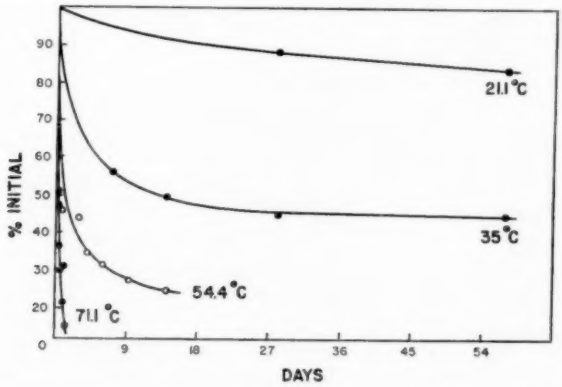

Figure 3. Decrease in the solubility of hematin pigments in freeze-dried beef during storage.

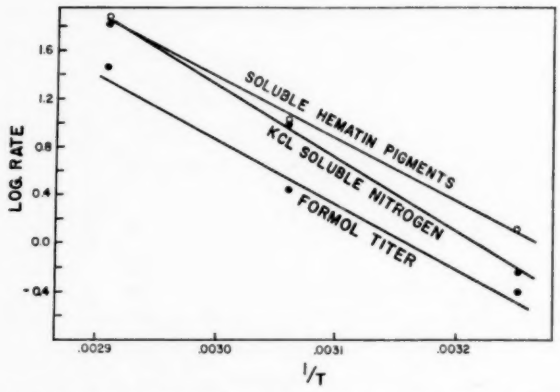

Figure 4. The effect of temperature of storage on the rate of change of three indices of deterioration of freeze-dried beef.

oxidase sample was low for it was incubated at room temperature for 3 hours before freezing, and some protein denaturation would have occurred during this time.

In no case was there a large change in the non-protein nitrogen. The biggest change was in the sample containing lactate which had a decrease of about 12% in the non-protein nitrogen in 16 days at 54.4° C.

Accelerated browning with additives. The visual browning scores for the samples of freeze-dried beef with various additives stored at 100° C. are shown in Table 1. The metabolic intermediates glucose and fructose-1,6-diphosphate, as well as the reductones, greatly accelerated the browning while the antioxidants had no significant inhibitory effect.

Effect of pH. The effect of pH on the rate of loss of soluble nitrogen was quite significant. The estimated initial rates are: pH 2.0, 0.1%/day;

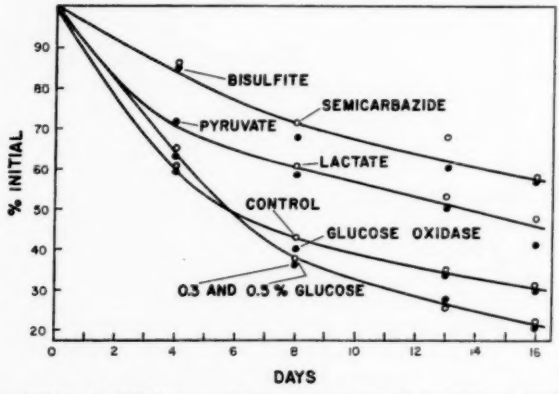

Figure 5. Effect of additives on the decrease of soluble protein of freeze-dried beef stored at 54.4° C.

TABLE 1

Accelerated browning of freeze-dried beef

Classification of additive	Additive	Visual browning score after 4.2 hr.
	None	2
Metabolic intermediate	4% Glucose	5
	10% Lactic acid	2
	10% Glycogen	1
	1% Fructose-1,6-diphosphate	3
Reductone	1% Ascorbic acid	5
	1% Morpholino-glucose reductone	5
	1% Piperidino-glucose reductone	3
	1% Dimethyl amino-glucose reductone	4
Antioxidant	0.5% Nordihydroguaiaretic acid	2
	0.5% Propyl gallate	3

pH 3.0, 0.2%/day; pH 4.5, 0.2%/day; pH 6.0, 0.8%/day; pH 7.5, 1.3%/day, and pH 9.0, 1.2%/day. The increase from pH 4.5 to 7.5 made the greatest change in the rate of deterioration.

Effect of volatile components. There was no apparent effect on the rate of deterioration by inclusion of the volatile trapping agents.

Effect of moisture level. The decreases in K Cl soluble protein of samples of freeze-dried beef of several moisture contents are shown in Figure 6. It is interesting to note that the deterioration proceeds quite rapidly at essentially zero moisture content (vapor pressure of solid magnesium perchlorate which is 5×10^{-3} mm. Hg).

DISCUSSION

It has been established that active carbonyl browning occurs during the storage of freeze-dried beef and is probably the only major—or at least the most significant—non-oxidative deteriorative mechanism *(15)*. The

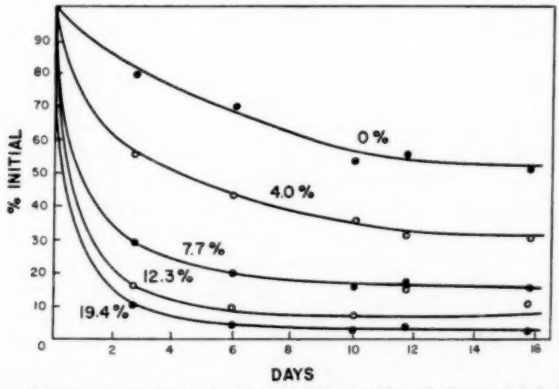

Figure 6. Effect of moisture on the soluble protein of freeze-dried beef during storage at 54.4° C.

possibility that heat denaturation of the proteins is also important is slight. Several of the changes noted during storage, such as the decrease in protein solubility and the increase in reducing compounds, are known to occur during the heat denaturation of many proteins in solution *(14)*. There is little information available in the literature on the heat denaturation of dry proteins. One may assume that the activation energy for thermal denaturation of dry proteins would be near that for denaturation in solution which is about 80 kcal./mole *(14)*. The results obtained by the three different analytical methods on samples stored at several temperatures give apparent activation energies of about 25 kcal./mole. This appears, then, to eliminate heat denaturation of the proteins as a major reaction in the storage deterioration of freeze-dried beef. Moreover, since the experimental value for the activation energy is in the range observed for browning in a variety of systems *(7, 10)*, this is further evidence that freeze-dried beef is undergoing active carbonyl-amine browning.

The fact that added glucose increased the deterioration while the carbonyl trapping compounds, bisulfite and semicarbazide, decreased the deterioration as measured by soluble nitrogen indicates that the carbonyl compounds, especially glucose, are the limiting reactants in the browning of freeze-dried beef. The small effect of the glucose oxidase may have been due either to a low enzyme activity in the preparation used or to a normal low level of free glucose compared to other active carbonyl compounds. In this respect it should be noted that the fructose-1,6-diphosphate increased the browning as measured visually in the accelerated browning study. All the samples with added reductones had greater browning than the control in the accelerated browning study. It is thus apparent that any normally occurring reductones will enter into the browning either as a primary reactant or an intermediate.

The effect of pH on the rate of loss of soluble nitrogen is fairly close to what would be expected of a browning reaction. Browning has been shown to be base catalyzed and acid inhibited; therefore, the rate would be low at low pH *(7, 10)*. The use of dry hydrogen chloride gas for the lowering of the pH of freeze-dried beef in the dry state has also been explored. The dry meat will readily absorb large quantities of hydrogen chloride. If enough hydrogen chloride is used so that the meat on rehydration is at pH 3.6, the meat has a dark brown color when cooked and is much more tender than the control at the normal pH 5.4. However, the flavor of the low pH meat is very poor, having a burned, rancid character. The possibility still exists for the use of a method such as this to reduce the pH to about 4.5, where the browning will be significantly decreased. At pH 4.5 there should be little or no hydrolytic effect during cooking.

In dehydrated meat such as used in these studies, the moisture level is so low that diffusion should be quite limited. In such a system one can visualize the possibility of some of the initial reactants or intermediates being relatively volatile. Thus the limitation on diffusion would be small and the reaction could proceed rapidly to completion. It has been recently shown that the deterioration of dehydrated pork produces volatile carbonyl compounds and basic compounds which are strongly reducing *(2)*. Similar compounds are probably formed in the deterioration of freeze-dried beef.

Our experiments using several trapping agents, however, showed no effect on the browning as measured by protein solubility. There are two possible explanations for these results. First, the trapping agents may not have been efficient in removing these compounds if they were actually entering into the reactions. Or, second, the volatile compounds found by Burnett *et al. (2)* may have been side products of the deterioration which cannot enter into further significant reactions. A trapping agent such as activated carbon can be of definite value for it removes the malodorous compounds formed during storage. These objectionable odors can be quite important in the acceptance of the stored product.

The observed activation energy for the deterioration corresponds to a Q_{10} of about 4. In general terms, this might be expressed as follows: An increase in the storage temperature of 10° C. would cause the storage life to decrease to one-quarter of its length at the lower temperature. This emphasizes the fact that no matter what other techniques may be used to increase the storage life of dehydrated foods, the control of the temperature should always be one of the foremost considerations.

The essentially complete removal of the water from freeze-dried beef does not completely eliminate the deterioration. However, above this minimum, the moisture level is quite important in determining the rate of deterioration. In-package desiccants can be used economically to increase storage life of freeze-dried beef. The drying rate after the meat has reached about 8% water is very low *(20)*. If a desiccant is included in the package and the initial storage is at a relatively low temperature, the material will reach a low moisture level and the storage deterioration will be significantly decreased.

The classical method of using high sulfite levels, as in dried fruits, is not applicable since meat is completely unacceptable under those conditions. However, the possibility for use of a very low level of sulfur dioxide in the gas used to release the vacuum in the chamber at the end of drying should not be eliminated. The amount of active carbonyl compounds in meat is fairly limited, and only a small amount of sulfite would be needed. Also, since the meat produced by this drying method is essentially raw after rehydration, most of the residual sulfite would be removed on cooking.

Since the level of the carbonyl compounds and the pH of the meat are so important in the storage deterioration, a possibility exists for the extension of the storage life by preconditioning the animal just before sacrificing. It has been shown that the pH of the meat can be significantly affected by the amount of exercise the animal has before being sacrificed *(1)*. Insulin shock could be used to decrease the concentration of glucose in the tissues prior to sacrificing, thus giving meat with a lowered level of one of the reactants in the deterioration.

Although glucose oxidase enzyme preparations have been shown to be effective in decreasing the storage deterioration of dehydrated ground pork *(6)*, its application to serving-size pieces of meat is not feasible. The diffusion of the enzyme into the tissue would be very limiting. However, if the enzyme were pumped into the whole carcass arterially, the distribution throughout the tissues might be adequate to give the desired decrease in glucose concentration.

SUMMARY

The apparent activation energy for the deterioration of freeze-dried beef during storage is 25 kcal./mole. This lends further support to the conclusion that browning is the major deteriorative reaction and eliminates the possibility of a significant amount of thermal denaturation.

The carbonyl compounds are the limiting reactants in the browning of freeze-dried beef. Lowering the pH causes a decrease in the rate of deterioration. The volatile compounds produced in the deterioration of freeze-dried beef apparently do not enter into further reactions, but are probably end- or side-products of the deterioration.

Essentially complete removal of water does not eliminate the storage deterioration. Moisture level is very important in the storage stability in that the rate of deterioration is increased with increasing moisture level.

Possible methods for increasing storage life of freeze-dried beef are discussed.

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REGENERATION AND STABILITY OF OXYMYOGLOBIN IN SOME GAMMA IRRADIATED MEATS[a]

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Meats irradiated with ionizing radiations often undergo pronounced color changes. These color changes have been noted and reported by many investigators *(6, 8, 9, 10, 11, 16)*. It is generally observed that meat irradiated in excess oxygen undergoes brown discoloration with the formation of metmyoglobin either immediately after irradiation or upon subsequent storage. With high irradiation doses, the hematin compounds may be destroyed oxidatively to a large extent. In the practical range of irradiation (0.05 to 3 megarep) these oxidative changes can be avoided by irradiation in inert atmosphere or high vacuum. When meat is radiated in inert atmospheres, a bright red coloration is commonly observed. This bright red coloration was noted by Hannan *(9, 10)*, Huber *et al. (11)* and others. Huber *et al. (11)* raised the doubt that this red pigment was oxymyoglobin but Schweigert *et al. (16)* and others have definitely reported it is oxymyoglobin.

It is the purpose of this paper to describe observations on the regeneration of oxymyoglobin by gamma irradiation of meat, to prove that oxymyoglobin is the bright red colored pigment of irradiated meat and to propose a chemical mechanism for its regeneration.

EXPERIMENTAL

Materials and irradiation. Most of the meat used in this study has been previously described *(8)*. The lamb was obtained from fresh leg of lamb, veal was obtained from fresh veal round steak, and turkey white meat was separated from the dark meat of fresh turkey. All meat was packaged and then was irradiated at the Materials Testing Reactor as previously described *(8)*. Purified methemoglobin was obtained from beef oxyhemoglobin by treatment with potassium ferricyanide and subsequent dialysis. The final concentration of methemoglobin at irradiation was 2.1×10^{-4}M in 0.1 M phosphate buffer, pH 7.

In this study, the samples used consisted of pork, beef, and chicken irradiated at 0, 0.7, 1, 2, 4, 8, 16, and 34 megarep, tuna irradiated at 0, 0.2, 0.5, 1, 2, 4 megarep, and lamb, veal, and turkey irradiated at 0.5, 1, 2, 4, 8, and 16 megarep. Pork and beef were irradiated at 3 megarep prior to 1 month storage at 70° F. and 95° F. The methemoglobin was irradiated at 2 and 8 megarep.

Spectral analysis. Visual observations of the irradiated meat were made immediately upon opening the cans. Subsequently, samples were prepared for measure of absorption spectra and portions of the meat were taken for reflectance spectra. For absorption spectra, the meats were blended 30 seconds in water in an atmosphere of

[a] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been approved for publication. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

air. Under these conditions, any myoglobin would be oxygenated to oxymyoglobin. Beef, veal, and lamb were used at a concentration of 25 g. of meat/250 ml. water because of the high concentration of hematin compounds. Tuna was used at 20 g./60 ml. water. Chicken and pork extracts of 50 g./150 ml. were used. Turkey extracts of 60 g./100 ml. were used. All extracts were allowed to settle by gravity and the aqueous portion was filtered. The extracts prepared in this manner from all irradiated samples and their controls were used for recording the entire visible spectra from 380 mμ to 650 mμ using a Beckman DR Recording Spectrophotometer. Reflectance spectra of irradiated tuna and pork were recorded using the reflectance attachment.

The complete visible spectra for the methemoglobin and irradiated methemoglobin were recorded. The alkaline pyridine hemochrome method *(13)* was used in determining destruction of the hematin nucleus in the methemoglobin.

The sensitive tannic acid test *(14)* and Katayama's qualitative test *(17)* for carboxymyoglobin were used. Carbon monoxide treated meat was used as a positive control.

RESULTS AND DISCUSSION

Spectral identification of oxymyoglobin as the bright red pigment. The visual observation showed that, in general, all meats irradiated in nitrogen atmospheres had a color which varied in intensity from bright pink to bright red. This red color was brightest for meat samples irradiated at 1-4 megarep. At the high irradiation levels of 16 and 34 megarep there was significant destruction of the hematin pigments as shown by color fading and spectral absorption. The most pronounced color difference was noted between the control tuna and pork and the irradiated tuna and pork. The color of the control tuna and pork was tan and tan-red, respectively, because of the formation of metmyoglobin. This formation of metmyoglobin is due to the oxidation of the myoglobin which occurred at the low oxygen partial pressures existing in these meats when packed in cans with a nitrogen atmosphere. A slight discoloration of the control was noted in the case of chicken and turkey but not for beef, veal and lamb. This discoloration to form metmyoglobin is apparently related to the low content of hematin pigments in tuna and pork and to oxygen consuming reactions such as lipid oxidation and oxidation of tricarboxylic acid cycle metabolites *(7)* which would tend to lower the oxygen partial pressure and deoxygenate the oxymyoglobin to myoglobin. This free myoglobin would readily oxidize to metmyoglobin.

In Figure 1 is given the spectral absorption of the extracts of control and irradiated tuna and pork. The spectral curve for the control tuna shows that it consisted entirely of metmyoglobin with inflection points at the maximum absorption of metmyoglobin at 630 mμ and 500 mμ. The spectral characteristics of the extract of tuna irradiated at 2 megarep is that of oxymyoglobin with the alpha band at 575 mμ, the beta band at 538 mμ and the Soret or gamma band at 418 mμ. These changes in spectral absorption show that metmyoglobin was converted to oxymyoglobin by irradiation. The spectral absorption of the extracts of pork show that the control sample contained large amounts of metmyoglobin as well as oxymyoglobin since it shows the absorption maxima at 630 mμ and 500 mμ of metmyoglobin and the absorption maxima at 580 mμ and 542 mμ of oxymyoglobin. The spectral absorption of the extract of pork irradiated at 2 megarep shows essentially complete conversion to oxymyoglobin. This study of pork irradiation also shows that the metmyoglobin is converted to oxymyoglobin by gamma irradiation.

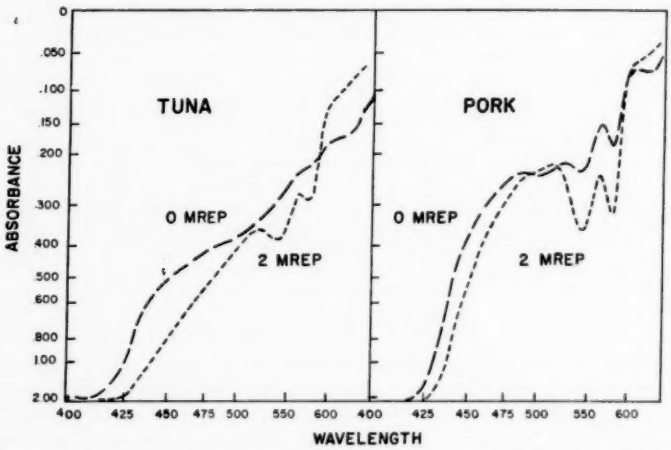


Figure 1. The absorption spectra of aqueous extracts of control and irradiated tuna and pork.

The wave length of maxima and minima absorption of extracts of a wide variety of control and irradiated meats are shown in Table 1. Although more extensive information is available on all of these meats at all irradiation levels used, the 2 megarep sample was chosen as being the most

TABLE 1

Wave-length of maximum and minimum absorption of extracts of meats

Source	Dose megarep	Maxima, wave-length, mμ			Minima, wave-length, mμ	
		α	β	γ, Soret		
Tuna	0	.....	.....	408	.....	.....
Tuna	2	575	538	418	562	523
Pork	0	580	542	410	565	.....
Pork	2	579	542	417	564	516
Chicken	0	582	544	412	568	517
Chicken	2	580	545	416	564	513
Turkey	0	580	544	413	563	512
Turkey	2	580	545	417	563	508
Beef	0	580	544	413	563	512
Beef	2	580	544	413	563	510
Veal	0	580	543	416	565	515
Veal	2	579	543	416	561	513
Lamb	0	583	545	418	565	515
Lamb	2	583	545	418	565	515
Pork, 1 mo., 70° F.	3	581	545	418	564	507
Pork, 1 mo., 95° F.	3	582	548	419	566	505
Beef, 1 mo., 70° F.	3	581	544	418	564	513
Beef, 1 mo., 95° F.	3	582	545	419	565	510
Oxymyoglobin *(3)*	0	582	544		564	510
Oxyhemoglobin *(15)*	0	576-578	540-542	412-415	563	512

interesting example for comparison with the control. In both tuna and pork there was a shift of the Soret peak to a maximum at a higher wave length; this being a characteristic of the conversion from metmyoglobin to oxymyoglobin. The same characteristic shift is noted for chicken and turkey both of which showed a smaller amount of regeneration of oxymyoglobin. In the case of beef, veal and lamb, the original oxymyoglobin was quite stable and there was no large amount of regeneration of oxymyoglobin. Oxymyoglobin in irradiated pork and beef which was subsequently stored at 70° F. or 95° F. for 1 month was stable as shown by its spectral characteristics in Table 1. A comparison of the maxima and minima for all of these irradiated meats shows that the bright red pigment is definitely oxymyoglobin because they have the same spectral characteristics as pure oxymyoglobin or oxyhemoglobin (given at the bottom of Table 1) and the same spectral characteristics as the oxymyoglobin in the control sample.

The use of reflectance spectra eliminates the possibility of creating pigment artifacts by aqueous extraction. It has the disadvantage of giving more diffuse spectra. The reflectance spectra of tuna and pork irradiated at 2 megarep in Figure 2 show all of the characteristics of oxymyoglobin.

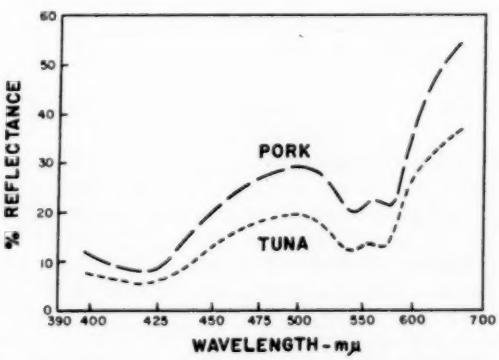


Figure 2. The reflectance spectra of irradiated tuna and pork.

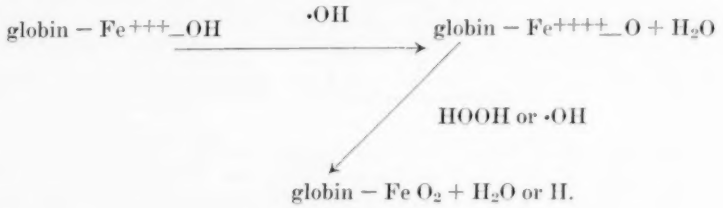
The reflectance spectra of irradiated tuna are similar to the absorbance spectra of aqueous extracts with maxima at 572 mμ, 540 mμ and 418 mμ and minima at 557 mμ and 498 mμ. The reflectance spectra of irradiated pork have maxima at 576 mμ, 542 mμ and 419 mμ and minima at 562 mμ and 502 mμ.

One possible source of error is that the formation of either nitric oxide myoglobin or carboxymyoglobin would both give a bright red color and would have similar spectral absorption. To eliminate this possibility of the formation of nitric oxide myoglobin and carboxymyoglobin a test was made using tuna and pork of the heat lability of the irradiated meat color. Upon cooking, both the pork and the tuna lost all red color and became the tan color of hematin. This shows the absence of heat stable nitric oxide hemochrome and carboxyhemochrome. The same observation has been consistently made in all cooked irradiated meat. Since the formation of any

carboxymyoglobin would be undesirable, this possibility was further evaluated using the sensitive tannic acid and Katayama's test both of which were negative on irradiated tuna and pork.

A proposed mechanism for the regeneration of oxymyoglobin. This proposed mechanism for the regeneration of oxymyoglobin from metmyoglobin is based on the following information. When pure methemeoglobin and metmyoglobin are reacted with large amounts of hydrogen peroxide they are oxidized to oxyhemoglobin and oxymyoglobin respectively *(12)*. In the catalytic decomposition of hydrogen peroxide by hematin compounds hydroxyl radicals appear to be intermediates as judged by the positive luminol test for their presence. George *(4, 5)* has obtained evidence that the reaction of metmyoglobin with hydrogen peroxide to give oxymyoglobin has ferrylmyoglobin as an intermediate.

The simplest mechanism visualized for the regeneration of oxymyoglobin is:



Globin – Fe^{+++} – OH diagrammatically represents the reactive species of metmyoglobin, the known starting material. Hydroxyl radicals are formed by the ionization of water by gamma radiation. Globin – Fe^{++++} – O represents ferrylmyoglobin, a proposed intermediate for which there is no direct evidence. Hydrogen peroxide is formed by a recombination of hydroxyl radicals and is a more likely reactant with ferrylmyoglobin than is hydroxyl radical alone. Globin – Fe^{++} – O_2 represents oxymyoglobin the known end product as shown by the evidence presented herein.

A test of the proposed mechanism. Purified methemoglobin in a nitrogen atmosphere irradiated at 2 megarep was converted to oxyhemoglobin as shown by its spectral characteristics given in Table 2. The yield of oxyhemoglobin was 8%. The major reaction products (> 90%) were insoluble hematin compounds and protein. The irradiation of dry hemoglobin with electrons or deuterons also leads to insoluble reaction products *(1, 2)*. Another deteriorative reaction caused by irradiating pure methemoglobin was the destruction of the hematin nucleus amounting to 76%

TABLE 2

Wave-length of maximum and minimum absorption of irradiated methemoglobin and pure oxyhemoglobin

	Maxima, wave-length, mμ			Maxima, wave-length, mμ	
	α	β	γ, Soret		
Methemoglobin, 2 megarep	570	536	417	562	513
Oxyhemoglobin *(15)*	576-578	540-542	412-415	563	512

at 2 megarep and 90% at 8 megarep. In this test, the identity of the control sample as methemoglobin was ascertained by its spectral absorption. Thus, the conversion of methemoglobin to oxyhemoglobin was caused entirely by gamma radiation. This evidence supports the postulated regeneration of oxyhematin compounds. The intermediate step in the proposed mechanism will be much more difficult to test because the exact mechanism of the reaction of pure hematin compounds with hydrogen peroxide and hydroxyl radicals has not been established although this subject has been under investigation for many years.

SUMMARY

Irradiation of meats containing metmyoglobin caused regeneration of oxymyoglobin. Oxymyoglobin in meats was stable to irradiation up to 8 megarep and to storage after irradiation.

It is proposed that the regeneration of oxymyoglobin involves the reaction of metmyoglobin with free radicals. Irradiation of purified metmyoglobin in aqueous solution produced some oxymyoglobin.

Acknowledgments

The author appreciates the assistance of Herman Groninger, Fred Knapp, and Vincent Maier.

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THE ONION: GASEOUS EMANATION PRODUCTS

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The problem of tracing normal food odors and off-odors to their source must ultimately be solved by actual identification and isolation of chemical components for individual study. To quote Turk *(11)*: "Much of our present information on constituents of common odors is purely nominal and based on old and uncomplete research." Such is the case for the onion.

Thus Semmler *(9)*, in 1892, claimed to have obtained a sulfide of unknown constitution from the onion, of the same molecular formula, but different boiling point than the allyl propyl disulfide which he reports in garlic oil. Kohman, *(7)* quoting Semmler as identifying allyl propyl disulfide as the onion flavor, measured this quantitatively by oxidizing the product of steam distillation with bromine to the sulfate state and determining sulphur gravimetrically. On the basis of the quantity of sulphur, allyl propyl disulfide was calculated. In an earlier paper Kohman *(6)* attributed the lachrymating properties of the onion to a thioaldehyde (thiopropionaldehyde or thioallylaldehyde) which he determined by the expedient of distilling the onion in vacuo at 50° C., oxidizing a weighed sample of the distillate residue with bromine and determining the sulphur present. The amount of sulphur was equivalent to that in the thioaldehyde which he hypothesized on the basis of the various observations made. Propionaldehyde was also identified by isolation and formation of its dinitrophenylhydrazone. Challenger and Greenwood *(2)* then detected n-propylthiol by absorption of the thiol in mercuric cyanide. They were not able to determine whether it occurs in the undamaged bulb or whether it is liberated by enzyme action after slicing.

Since little positive information has been published concerning the volatile constituents of onions, it was decided to apply the methods of instrumental analysis with the intention of outlining a technique which may be applicable to most other food odor problems. The following work is a report on a mass spectrometric and infrared spectroscopic analysis of vapors trapped at various low temperatures in the absence of air.

EXPERIMENTAL PROCEDURE

A model 21-103B Consolidated analytical mass spectrometer and a Baird infrared spectrometer were used in the present work. Volatile constituents of onions were collected by trapping the vapors at −30°, −80°, and −190° C. in a system as shown in Figure 1. The contents of the traps were individually analyzed mass spectrometrically, and then condensed into the distillation tube, or examined by gas partition chromatography (GPC). The distillation technique yielded between 3 to 5 cuts from the contents of each trap. A trapping system made possible the collection of gases separated by GPC.

[a] Presently with the Bakelite Research Center, Bloomfield, New Jersey.

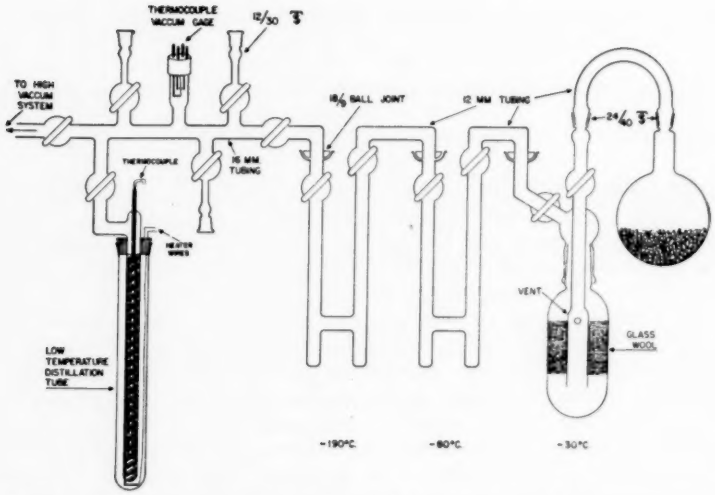

Figure 1. High vacuum trapping system and low temperature–low pressure distillation tube for prefractionation.

Preparation of system. Approximately 100 to 140 g. of onion are frozen in liquid nitrogen, minced, and placed in the 500 cc. distillation flask (Figure 1). Liquid nitrogen coolant is placed around the flask while the system is evacuated to less than 0.1 micron of mercury. The flask is isolated from the traps, and the onion is permitted to warm to room temperature. A heat lamp or water bath may be used to speed the warming process provided care is taken to prevent overheating the sample. At no time should the sample temperature exceed 30° C. It was found that slow cooling of the onion at this time to –30° C. ruptures the cells and yields a greater concentration of volatile matter.

Collection of vapors. Coolant is applied to the first trap and the onion is permitted to warm up and begin to distill into the first trap. The second and third traps are likewise cooled and stopcocks are opened to permit collection of the volatile emanations of the onion. The "H" type trap was used for the latter stages of trapping in order to maximize the cooling surface to volume ratio. The function of the glass wool used in the first trap was also to create a greater effective cooling surface for this stage, and to entrain micro-particles of frozen water.

It should be noted that the system may be constantly evacuated or isolated from the vacuum source as desired while collecting sample. Both techniques were employed, but it was found that the latter provides cleaner separations especially with respect to water carryover, whereas the former technique yields a greater concentration of collected vapors.

At least eight hours are required to provide adequate samples for analysis, and overnight runs usually dehydrate the onion sufficiently to provide more than enough condensate.

A portion of the vapor phase contents of each trap is analyzed directly on the mass spectrometer and the residual is condensed into the distillation tube or separated by vapor phase chromatography. The amount used for mass spectral analysis is quite small (approximately 5×10^{-5} moles) and does not represent serious reduction of sample size.

The distillation tube. The tube itself is an adaptation of a design originally used by the Bureau of Standards as an isothermal distillation tube *(10)*. A copper constantan thermocouple measures the internal temperature in the bottom of the distillation tube; the column is packed with stainless steel balls, and a nichrome ribbon is wrapped

around the tube to supply the heat necessary to conduct a distillation. A variac is used in series with a dropping resistor in a 115 V AC power source to supply approximately 15 to 20 watts to the heater, depending upon the speed of distillation desired. It is advisable to allow at least 6 hours for a single distillation from −190° C. to +25° C. The tube has a capacity for approximately 1 ml. of liquid, but less material than this is desired since only 1 to 5 mm. mercury of vapor is collected in a 250 ml. gas sampling bottle for further analysis. Normally the sample size is too small for a liquid-vapor phase equilibrium to exist in the column, so the purpose of the column as used herein is to allow the vapors to fractionally diffuse through the packing. The gas bottle is equipped with an oblique bore high vacuum stopcock and a 12/30 standard taper joint.

Distillation. After analyzing the contents of one of the traps mass spectrometrically, the trap is attached to the high vacuum manifold and is cooled with liquid nitrogen. The system is evacuated and a bulb to bulb distillation condenses the contents of the trap into the lower portion of the tube. The entire contents of the −80° and −190° traps are condensed into the tube, but since approximately 85% of the mass of the onion is collected in the −30° trap, it is necessary to obtain a small aliquot from this trap for distillation. A fraction is collected when the pressure in the system exceeds 200 microns of mercury, and the pressure does not appreciably rise with increasing temperature. Needless to say, the contents of each trap is distilled separately. The fractions are then analyzed mass spectrometrically. Table 1 illustrates the results obtained by applying this technique to an onion fraction.

TABLE 1

Contents[1] of a −190° C. trap analyzed before and after separation by use of the distillation tube

	Mole per cent					
	Before distillation	After distillation				
Temp. degrees C.	+25	−117	−95	−90	−42	+25
Propyl mercaptan	0.1	0	0	0	1.0	0.6
Propionaldehyde	21.2	3.3	1.3	0.1	53.1	21.4
Acetaldehyde	0.8	0	0	0	42.3	0.1
Carbon dioxide	65.8	94.1	98.1	99.8	1.5	0.1
Methyl alcohol	12.1	0.9	0.5	0.1	2.1	77.8
Hydrogen sulfide	0	1.7	0.1	0	0	0

[1] Calculated air and water free. Zero values indicate less than 0.05% detectable.

Gas partition chromatography. A column (10 mm × 2 meter), packed with Celite 545 having diethyl hexyl sebacate as the non-mobile phase, was used with a Gow-Mac thermal conductivity cell as the detector. A flow rate of 50 ml. per minute of helium was maintained, and the column was thermostated at 25° C. The gas phase of each trap was condensed into a special inlet system designed to minimize the volume of the mixture. The sample was then vaporized and introduced into the column by displacement with helium. Fractions were collected of each peak appearing on the chromatogram and analyzed mass spectrometrically.[b] Figure 3 depicts a gas-partition chromatogram obtained, with subsequent mass spectral analysis of each fraction presented in Table 2.

Mass spectrometric analysis. The gas bottles containing the various fractions obtained by distillation or by chromatographic separations are introduced into the mass spectrometer and a record is obtained which represents the fragmentation pattern of the different molecules present in the sample. By applying classic techniques it is possible to identify the components present from their fragmentation pattern. It is not the intention of the authors to discuss in greater detail the theory, calculations of data,

[b] This technique is described in detail in a paper submitted to *J. Agr. Food Chem.* by Stahl, Niegisch, and Levy.

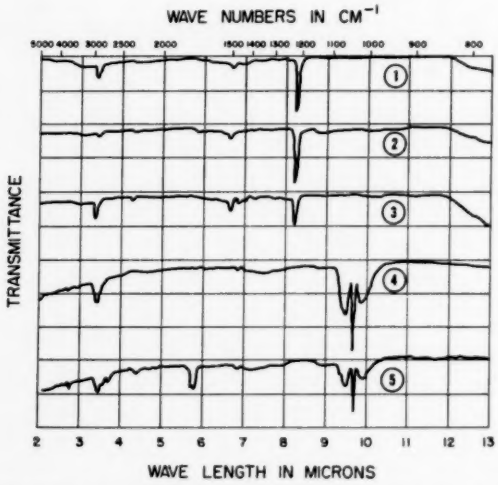

Figure 2. Infra-red spectra indicating the presence of n-propyl mercaptan and methanol in onions. Top to bottom: 1) 0.05% propyl mercaptan in CC14; 2) CC14 extract of onion; 3) CC14 extract of water solution collected in −20° C. trap from domestic yellow onion; 4) Methanol vapor phase; 5) −190° trap contents of Spanish onion, vapor phase.

or operation of the mass spectrometer. The literature has excellent articles which discuss the subject in great detail *(3, 8)*. By operating the instrument at high sensitivity it is possible to detect less than 5×10^{-7} moles of a particular molecular species.

Infrared analysis. Considerable difficulty was encountered in detecting small quantities of material present in addition to water in the −30° C. trap because of the preponderance of water. A carbon tetrachloride extract of the contents of this trap was analyzed in a 0.1 sodium chloride cell against a carbon tetrachloride reference cell. The resulting spectrum is shown in Figure 2 and may be compared with a carbon tetrachloride extract of a whole Waring blended onion, and a spectrum of 0.05% n-propyl mercaptan. It is seen that the characteristic frequencies related to n-propyl thiol are found in the onion samples examined. The vapor phase contents of a −190° C. trap is also shown in Figure 2 and comparison with a spectrum of methanol indicates the presence of methanol in the trap. A 10 cm. sodium chloride cell was used for this analysis.

TABLE 2

Contents[1] of a −190° C. trap analyzed before and after separation by vapor phase chromatography

	Mole per cent				
	Before separation	After separation			
Cut number		1	2	3	4
Propyl mercaptan	0	0	0	0	0.1
Propionaldehyde	3.2	0	0	0	89.9
Acetaldehyde	2.3	0	98.4	6.3	1.6
Carbon dioxide	93.7	100.0	1.6	1.7	0
Methyl alcohol	0.8	0	0	92.0	8.4

[1] Calculated air and water free. Zero values indicate less than 0.05% detectable.

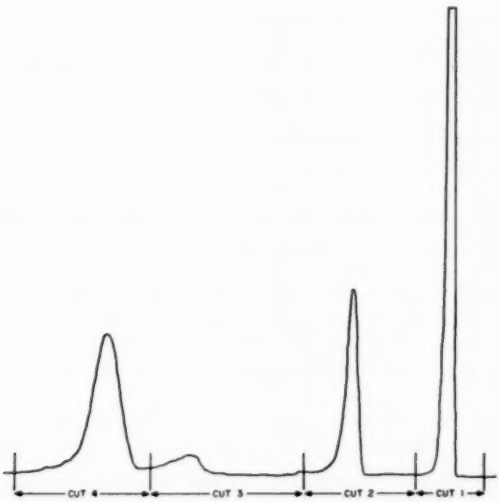

Figure 3. Chromatogram of the contents of a −190° C. trap fraction. (See analysis of cuts obtained in Table 2.)

Adsorption. An attempt was made to employ various adsorbants such as Drierite, Ascarite, Linde Molecular Sieves, alumina, etc., to facilitate the removal of the large amounts of carbon dioxide and water which tend to mask the detection of the odoriferous onion components. It was found by passing the contents of a −80° C. trap over a column of potassium hydroxide pellets, that the exit gases contained a small amount of di-(n-propyl) disulfide.

RESULTS AND DISCUSSION

From the data presented in Figure 2 and by mass spectral analysis, the presence of n-propyl mercaptan and methyl alcohol is unequivocally established. Table 2 also conclusively proves the presence of propionaldehyde, acetaldehyde, and carbon dioxide by virtue of the relatively large concentrations found in the chromatographic cuts (see Figure 3) when analyzed mass spectrometrically, and by calibrating compounds by gas-partition chromatography (not shown).

Because of the ability of the mass spectrometer to detect very small amounts of material in the presence of large diluents under certain conditions evident to a mass spectrometrist, the detection of other materials is reported below, although no verifying data applying other techniques is offered. Extreme caution was exercised by the authors to eliminate all evident sources of possible contamination, and thus to permit the claim that the following are also true components of onion: propyl alcohol, sulphur dioxide, hydrogen sulfide and di(n-propyl)disulfide. The latter has a definite "sweet" odor typical of disulfides, and when combined with n-propyl mercaptan, a creditable onion odor is produced.

Theoretical predictions. By applying the technique of structural analysis to the mass spectral scans, it is postulated that a hexenal may also be present in onions. Structural deductions drawn from a composite spectrum

calculated from more than 20 scans of various onion fractions indicated that the compound may be 4-hexen-1-al. It is certain that the unknown is not the "leaf aldehyde" (2-hexen-1-al) discussed by Guenther *(5)* because of the dissimilar mass spectra of these two compounds. It was also ascertained that the unknown is not α-methyl, β-methyl acrolein, which is the condensation product of propionaldehyde, and was first suspected, but also eliminated because of differences in the ionization fragmentation pattern.

Kohman *(6)* suggested that the lachrymatory principle of the onion was due to a thiolaldehyde. There was no evidence from any data obtained in this investigation that either thiopropionaldehyde or thioallylaldehyde was collected in any of the traps. However, a compound of mass 90 has been observed several times in the −80° traps which is known from isotopic measurements to contain one sulphur atom in the molecule. There are eight obvious mass type isomers containing carbon, hydrogen, sulphur and/or oxygen atoms which would have a mass of 90. Molecules containing nitrogen atom were eliminated because those which have one nitrogen atom have uneven molecular weights, and compounds containing more than one atom of nitrogen and one sulfur atom do not have molecular weights of 90.

Isomers of Mass 90 Containing Sulfur

1. $CH_3 - \overset{\overset{O}{\uparrow}}{S} - CH_2 - CHO$
2. $CH_3 - \overset{\overset{O}{\uparrow}}{S} - CH - CH_2$
3. C_4H_9SH
4. $R - S - R'$ $(R + R' = \text{MASS } 58)$
5. $CH_2 = CH - CH_2 - \overset{\overset{O}{\|}}{S}H$
6. $CH = CH - \overset{\overset{SH}{/}}{\underset{\underset{OH}{\backslash}}{C}}\!-\!H$
7. $HS - CH_2 - CH_2 - CHO$
8. $HO - CH_2 - CH_2 - CHS$

The spectrum containing the mass 90 unknown is also characterized by the presence of mass 73 and 45 which are not common breakdown masses. They could conceivably arise from cleavage of a hydroxyl group to give a mass 73, and either a carboxyl, $-CSH$, $-CH_2CH_2OH$, $-CH_2-O-CH_3$, $-OCH_2-CH_3$ or $-\overset{\overset{H}{|}}{C}=S$ to produce a mass 45 fragment. It is readily seen that the first four compounds above do not meet both these requirements. Structural analysis also eliminates the presence of an ether linkage. Compound five, allyl sulphenic acid, has never been isolated and is usually considered an intermediary indicating oxidation of a disulfide to a sulfinic acid. It is highly unlikely that this compound is present from the existing information regarding sulfenic acids.

Attempts to synthesize compound six have failed. However, the hemithial of propanionaldehyde, $CH_3 - CH_2 - \overset{\overset{SH}{/}}{\underset{\underset{OH}{\backslash}}{C}}\!-\!H$, was readily prepared by the addition of hydrogen sulfide to propionalaldehyde (80% yield). The structure was deduced from information obtained by infrared observation

of the decrease of the carbonyl band and formation of water accompanied by increasing cloudiness of the solution on standing. This observation would imply the addition of hydrogen sulfide to form the hemithial, with subsequent release of water and trimerization of the resulting propanthial. **Analysis of the mixture mass spectrometrically** showed no ionization to mass 90 although the data obtained by infrared indicated 80% of the hemithial present. Instead, only water, hydrogen sulfide and propionaldehyde were found. This indicates the instability of the hemithial in the high vacuum (10^{-6} mm mercury) of the mass spectrometer ionization chamber. The hemimercaptal prepared in this Laboratory by the reaction of acetaldehyde and methyl mercaptan analyzes similarly by infrared and mass spectrometric techniques. It is assumed that the hemithial of acrolein, structure six, will behave in a similar manner in the mass spectrometer, and will never ionize to the molecular weight mass. It is interesting to note that the hemithial of propionaldehyde has a strong persisting onion-like odor.

Compound seven, β-mercapto propionaldehyde, has been reported to have a definite onion-like odor *(4)* and was suspected as an onion constituent. The predicted fragmentation pattern for this compound coincides generally with the postulated fragmentation pattern of the unknown, except for the lack of a hydroxyl group which would account for a mass 73 breakdown. Compound eight, β-hydroxy propanthial, satisfies all of the requirements of a predicted fragmentation for the unknown. In addition, this compound would be expected to polymerize readily, accounting for the low concentration of this material present in the volatile onion vapors collected. It is conceivable that the allyl thial predicted by Kohman could easily add water to produce the β-hydroxy propanthial postulated in this investigation.

As it is well known that mercaptans oxidize readily to disulfides, it was deemed necessary to show that dipropyl disulfide found could not be formed under the conditions of isolation existing in this investigation. Therefore, n-propyl mercaptan was deliberately diluted to contain 20% air, and the mixture was run through wet potassium hydroxide, which were the conditions existing during the initial detection of the disulfide. No trace of the disulfide was found upon analysis of the scrubbed gases.

All onions used in this work were the common domestic yellow with the exception of one determination made on a Spanish onion from which the infrared data establishing the presence of methanol was shown. It was found that the domestic yellow generally produced the greatest amount of condensate other than water and carbon dioxide.

All data presented have been obtained in the absence of air at room temperature, and samples were analyzed entirely at very low pressures to reduce the possibility of oxidation, reduction or other chemical changes which may take place. Vapors were condensed and stored at low temperatures, which would retard the physical changes which may take place under conditions existing in other investigations. All data were obtained within 48 hours or less after collection of sample. This would suggest that all of the compounds found, exist *per se* in the onion in contrast to the data of Buck and Joslyn *(1)* wherein they suspect the source of acetaldehyde in

broccoli to be brought about by enzymatic decarboxylation after long term storage in the frozen state.

It is difficult to assay the data obtained quantitatively, yet something should be said as to the relative concentration of each material reported. The following is a relative estimation of each component present, in decreasing order of concentration. The gross amounts of carbon dioxide and water are not included in this tabulation.

Very abundant	Abundant	Small	Trace
CH_3OH	$CH_3-CH_2-CH_2SH$	H_2S	SO_2
CH_3-CH_2-CHO		CH_3-CHO	$C_3H_7-S-S-C_3H_7$
			C_3H_7OH
			$CH_3-CH=CH-CH_2-CH_2-CHO$[1]
			$HOCH_2-CH_2-CHS$[1]

[1] Suggested structures.

CONCLUSION

It is evident from the data presented in this paper that the analysis of food odors is greatly simplified by applying modern instrumentation to a quantity of starting material many orders of magnitude less than that required by classic chemical methods. In addition, it is particularly well suited to the analysis of gases and vapors which are commonly overlooked by other methods. It may be suggested to the laboratory only set up for classical chemical methods that all of the instrumental techniques presented need not be applied simultaneously. Indeed, infrared data on GPC or distillation cuts can be obtained quite successfully without mass spectrometer analysis provided larger samples are obtained.

There can also be no ambiguity as existed in the reports of earlier investigators. The authors make no claim that this work reports all of the volatile odors emanating from onions. However, no evidence of the existence of allyl propyl disulfide reported by other investigators was found despite the great care taken in processing samples and searching for this compound. It is very likely with the advent of greater sensitivity of gas partition chromatography that better separations will be possible in the near future which will facilitate the detection of materials present in extremely small concentrations.

Acknowledgments

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INFLUENCE OF VINING ON THE DEVELOPMENT OF OFF-FLAVOR IN FROZEN RAW PEAS[a,b]

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(Manuscript received July 26, 1956)

While it has been known that unblanched peas deteriorate progressively during storage at –17.8° C., it was observed, however, that frozen unblanched peas held in storage in pods seemed to retain higher quality. The present study was undertaken to determine whether such retention of quality was imaginary or real.

MATERIALS AND METHODS

Thomas Laxton peas, grown on the Experiment Station farm, were harvested at a tenderometer reading of 113, processed, and stored as follows. Peas to be frozen in the pods but otherwise untreated, were harvested from the field at random by hand. The same field was then mechanically harvested after which the plants were vined mechanically. The vined peas were cleaned, during which operation the unusable peas, smaller than size No. 1, were removed, and divided into two lots. One lot was washed, sorted, and packed in 10-pound tins ungraded as to size. The second lot was washed, blanched in boiling water at 100° C. for 60 seconds, cooled in very cold water, sorted, but ungraded as to size, and packed in 10-pound tins. The peas in the pods were packed in 30-pound tins. All samples were frozen and stored at –17.8° C. Organoleptic tests were run on the freshly harvested peas after cooking *(4)*, by a competent taste panel. A scoring system of 1 *(very poor)* to 10 *(excellent)* was used. Judges were not advised as to the treatments until the tests were concluded. In addition to this, fresh samples were frozen, lyophilized, extracted with anhydrous peroxide-free ethyl ether and the extracted crude lipid material assayed for acid and peroxide numbers *(2, 3)*. Samples were removed from the stored material at regular intervals for similar taste tests and chemical examination. In addition to the tests for changes in the lipid material, samples were taken for determination of the reducing sugars and sucrose by the Munson-Walker method *(1)*. A further examination was made for total chlorophyll in the several samples after a suitable storage period *(5)*.

RESULTS AND DISCUSSION

Results of this work are shown in Tables 1, 2, and 3, and in Figures 1 and 2. Table 1 shows the results of the taste tests on the several samples, as well as the development of acids and peroxides as determined by analysis of the extracted crude lipids. In addition, Figures 1 and 2 show the chemical data graphically.

Table 2 gives the data obtained on sugar changes, and Table 3 gives that obtained on changes in chlorophyll.

It is obvious from an inspection of these data that acid changes found on analysis of the extracted crude lipids from vined peas are reasonably close to those obtained by analysis of the extracted crude lipids prepared from the same lot of peas which had been held in frozen storage in the pods.

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[a] Presented at the Sixteenth Annual Meeting of the Institute of Food Technologists, St. Louis, Missouri, June 13, 1956.

[b] Approved for publication by the Director of the New York State Agricultural Experiment Station as Journal Paper No. 1043.

TABLE 1

Storage tests on frozen Thomas Laxton peas,[1] 1955 harvest (storage temperature −17.8° C.)

Period of storage days	Frozen in pods (shelled previous to analysis, pods discarded)					Frozen after vining in commercial viner					Blanched 60 seconds in boiling water at 100° C.				
	Taste tests			Chemical tests		Taste tests			Chemical tests		Taste tests			Chemical tests	
	F*	T**	C***	Acid No.	Peroxide No.	F	T	C	Acid No.	Peroxide No.	F	T	C	Acid No.	Peroxide No.
0						7.8	7.6	8.4	10.2	0	5.4	6.4	7.5		
5				18.9	0										
9	7.5	7.6	7.6			6.3	6.2	6.9			6.4	6.6	7.4		
13				33.8					22.6	0				6.7	0
14	7.8	7.5	7.1			6.3	6.9	7.5			6.1	6.9	8.2		
20	7.6	7.9	7.1	37.2	0	5.5	7.0	7.0	31.9		6.7	6.5	8.0		
27				39.8	0				30.9	0					
28	6.8	6.5	6.9			5.5	6.4	6.0			5.9	7.0	8.1		
34	6.7	7.1	6.1	43.1	0	4.7	5.5	6.3	43.1	0	6.7	6.6	8.1		
41				33.6	0				36.9	1.7					
42	7.1	7.1	7.4			5.3	6.2	6.3			6.6	7.0	8.3		
47				54.8	0				51.5	0					
48	6.0	7.4	6.9			4.5	6.2	6.3			5.9	6.4	8.6		
55	6.4	7.1	5.4	57.0	1.6	3.1	6.1	5.7	55.6	2.6	6.1	6.2	8.6		
62	5.6	7.2	5.4	43.9	2.1	4.2	5.5	5.4	54.1	8.6	6.2	6.4	8.1		
69	5.5	7.2	5.2	52.9	3.7	3.6	5.7	4.7	51.4	2.7	6.9	6.2	8.2		
76	5.7	6.1	4.8	66.8	5.5	3.7	5.9	5.4	62.0	1.9	5.9	6.2	8.3		
83	5.3	6.6	4.7	67.0	19.6	3.3	5.8	5.1	69.8	5.5	6.1	6.0	7.9		
97	5.1	6.8	4.1	68.6	22.3	2.7	5.3	5.4	67.3	2.1	6.2	6.6	8.3		
125	5.0	6.6	5.5	62.4	23.5	3.6	6.4	5.5	57.2	3.7	7.0	6.8	8.4		
153	4.8	5.7	3.8	78.5	41.0	3.0	5.0	5.0	78.1	5.8	6.1	6.5	8.2		
205	3.9	5.9	2.9	75.3	10.1	2.4	5.6	4.6	71.7	12.3	6.8	6.9	8.2		
334	3.5	6.2	4.0	72.3	19.8	3.0	6.3	5.7	70.2	Trace	6.6	6.6	9.3		
338														5.5	0

*F Flavor **T Texture ***C Color

[1] Tenderometer reading 113.

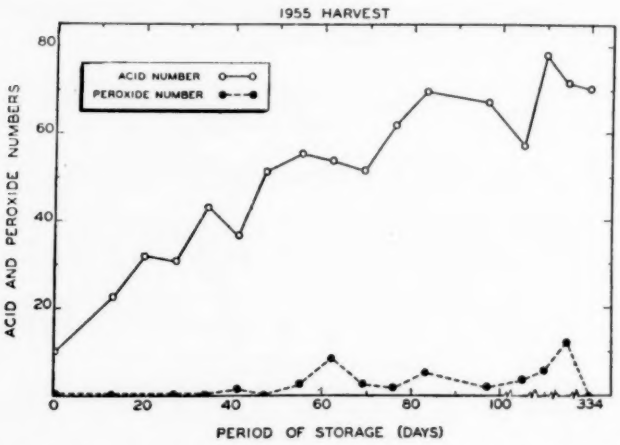

Figure 1. Chemical tests on vined peas during storage at −17.8° C. (Thomas Laxton variety).

The increase was progressive, as found during previous work *(4)*. The starting figure was a little higher than that obtained for the blanched material, but these figures are within reason.

The figures obtained for the changes in peroxide number of the extracted lipids show an entirely different picture. As found in previous work *(4)*, the development of peroxides as shown by analysis of the extracted crude lipids showed a varying trend characterized by low values as long as these tests were run. In the past, work in this laboratory showed higher peroxide values with material of this character after longer than a

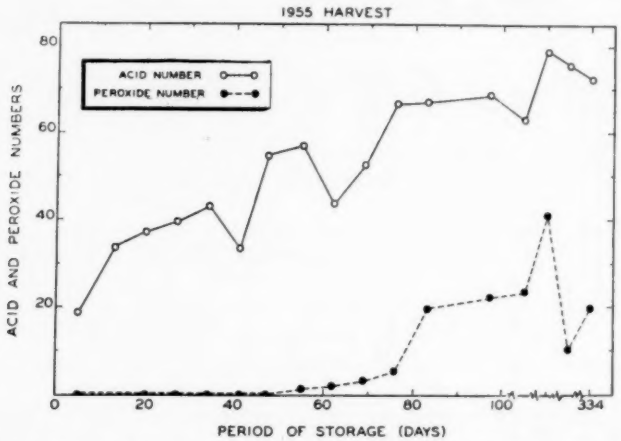

Figure 2. Chemical tests on peas in pod during storage at −17.8° C. (Thomas Laxton variety).

TABLE 2
Sugar contents of frozen Thomas Laxton peas[1] held in storage at –17.8° C. 1955 harvest

Period of storage days	Frozen in pods (shelled previous to analysis, pods discarded)			Frozen after vining in commercial viner			Blanched for 60 seconds in boiling water at 100° C.		
	Reducing sugars	Sucrose	Total sugar	Reducing sugars	Sucrose	Total sugar	Reducing sugars	Sucrose	Total sugar
	%	%	%	%	%	%	%	%	%
0	Trace	5.28	5.28	Trace	5.28	5.28			
38	0.09	6.01	6.10	Negative	5.26	5.26			
83	0.29	5.80	6.09	Trace	5.32	5.32			
160	0.30	5.94	6.24	Negative	5.09	5.09			
209							Trace	4.28	4.28

[1] Tenderometer reading 113.

year of storage at –17.8° C. The peroxide values obtained from crude lipid material extracted from peas held in frozen storage in the pods showed an entirely different result. After the 62nd day of storage, the peroxide values obtained from crude lipid extracted from this material exceeded those obtained from the corresponding vined samples of peas. On the 153rd day of storage, the highest value for the peas stored in pods was obtained, namely, 41.0. The peroxide values obtained for the two subsequent tests, after 205 and 334 days of storage were 10.1 and 19.8, respectively.

It is curious, also, that the flavor of these peas stored in the pods held up particularly well for 42 days, after which a gradual deterioration was noted. The flavor of the peas which were vined previous to storage showed evidence of deterioration at the time of the first storage test which took place after nine days at –17.8° C. The flavor tests on the vined peas gave lower values throughout the series of tests.

Results of the sugar determinations are particularly interesting. It can be seen from an inspection of Table 2 that total sugars are higher by almost 1% in the peas stored in the pods than in those which were vined before placing in storage. Blanched peas show the smallest amount of total sugars. This latter result is to be expected because of losses of soluble materials during blanching. Another result worthy of note is the presence of detectable amounts of reducing sugars in the peas stored in the pods. Reducing sugars in the vined peas were either absent or present only in traces. When the samples were being scored organoleptically, most of the judges noted that the samples which had been held in storage in the pods were slightly sweeter in taste than the others.

TABLE 3
Chlorophyll content of frozen Thomas Laxton peas[1] held for 160 days in storage at –17.8° C., 1955 harvest

Condition of sample	mg./100 g. fresh weight	mg./100 g. dry weight	% loss, fresh weight basis	% loss, dry weight basis
Blanched	9.42	41.77	0	0
Stored in pods (shelled previous to analysis, pods discarded)	6.04	26.95	35.9	35.5
Raw, vined	6.87	31.44	27.0	24.7

[1] Tenderometer reading 113.

It was obvious on inspection that after fairly long periods of storage the color of the peas stored in the pods had undergone greater deterioration than those vined previous to storage. This can be seen from the data given in Tables 1 and 3. The latter table shows that larger chlorophyll destruction occurred in peas stored in the pods.

The reason for these differences are obscure at the moment, but it is suggested that since the peas stored in the pods are injured the least previous to storage, perhaps areas in which peroxides are formed contain little material on which these peroxides can act, thus resulting in an accumulation of peroxides. The peroxides perhaps are an intermediate in the formation of off-flavors. The fact that the peas in the pods are really being stored in gas chambers may have a bearing on the observed sugar differences, and possibly on the peroxide and chlorophyll differences as well.

The authors do not wish to convey the impression that the storing of unblanched frozen peas in the pods or otherwise is recommended. This is basically a part of a fundamental study of the biochemistry of frozen peas, rather than a practical application.

SUMMARY

A comparison was made which involved the chemical and organoleptic differences in unblanched peas vined previous to storage at –17.8° C., and those stored in the pods. A control on blanched material was run also.

It was found that peroxide values of the extracted crude lipids obtained from peas stored in the pods longer than 62 days were considerably larger than those found in the crude lipids extracted from peas which were vined previous to storage.

Total sugars, reducing sugars, and sucrose were higher in the peas stored in the pods than in those which were vined previous to storage.

Greater chlorophyll degradation took place in the peas which were stored in the pods than in those which were vined previous to storage.

Suggestions were made as to the possible causes of these phenomena.

Peas stored at –17.8°C. unblanched in the pods retained reasonably good eating quality for a little over a month. After this period gradual deterioration became apparent. Unblanched vined peas started to decline in quality after about a week of storage.

Acknowledgment

The authors wish to thank Barbara Young for assistance in some of the analytical work required in this study.

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FEEDING STUDIES ON VITAMIN- AND MINERAL-FORTIFIED WHOLE MILK[a]

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(Manuscript received July 28, 1956)

The first fluid vitamin fortified milk was introduced on the market just 25 years ago by the Walker Gordon Farms in Plainsboro, New Jersey. This was a vitamin D fortified milk produced by feeding irradiated standardized yeast to a herd of certified cattle. The milk was known as certified (metabolized) vitamin D milk. Following this, other types of vitamin D milk were introduced such as Irradiated Vitamin D (direct irradiation) Milk and the Concentrate type where the highly purified standardized vitamin D was added directly. Of the three processes above mentioned, there now remains only one of practical importance, and that is the concentrate method of fortification, as it is the one of choice in the dairy industry and subject to the greatest control from most standpoints.

Methods of fortification in use today in all branches of the dairy industry employ concentrates of the vitamin which are added directly to the milk. This process is referred to as direct fortification.

Health authorities at one time had some degree of skepticism about the addition of vitamin concentrates directly to milk; however, such objections have now virtually disappeared. The commonly held concept that milk could or should not be adjusted or modified in any manner has given way with further advances in processing; newer findings revealed that milk could be improved, and such improvement would result in better health for the user.

For several years vitamin D fortified milk was the only product in the fresh milk industry that was vitamin fortified. However, with establishment of the fact that this was a sound public health practice, attested to by the virtual disappearance of rickets from the country, other improvements in the enrichment of milk were under way. Another meritorious move successfully undertaken was the addition of vitamin A and vitamin D to skimmed milk. The latter product, employed in slimming diets, is practically devoid of these two vitamins, and much benefit may accrue by fortification in the case of vitamin A to at least the maximum value found in whole milk, and in the case of vitamin D to the 400 units per quart potency carried in all vitamin D milks—thus assuring proper utilization of the calcium in which milk so richly abounds.

Some 12 years ago there was developed a formula to be added to milk and referred to as DQ, meaning Daily Quota in the quart of milk fortified with this formula. This product was introduced in two test markets: Milwaukee, Wisconsin, and Bridgeport, Connecticut. The formula consisted of 5 vitamins and 2 minerals, the purpose of which was to further balance and

[a] Presented at the Sixteenth Annual Meeting of the Institute of Food Technologists, St. Louis, Missouri, June 11, 1956.

improve the diet by means of this more nearly balanced fortified milk. Careful observation of this vitamin and mineral fortified (VMF) milk over the course of many months in the two test markets indicated that the product was well accepted and filled a definite need. Following this, distribution of the VMF milk was gradually expanded until today it is to be found in well over two-thirds of the 48 states. However, as the sales of VMF milk expanded and the growth of the product continued, certain questions were raised in some areas relative to the nutritional safety of a milk fortified with extra vitamins and minerals. It was at that time that the Wisconsin Alumni Research Foundation was approached to investigate these questions as to nutritional safety and to conduct the series of feeding studies reported herein. The basic idea was to carry this work through successive generations of rats to observe the effects on such factors as growth, reproduction, tissue changes, and general appearance which could be attributed to the fortification.

A great deal is known about the effects of vitamin deficiency or of huge intakes of a particular vitamin. Comparatively few data are available on the effects of moderate vitamin (and trace mineral) supplementation over and above minimum requirements for growth and physiological functions. In the present studies rat feeding tests were performed on commercially available vitamin-mineral concentrates designed for fortifying whole milk.

Difficulties that are likely to be encountered in subjecting human diets to rat tests are well summarized in the book *The Appraisal of Human Dietaries by Animal Experiments (7)*. The experiments reported herein were designed after consultation with a number of leading authorities on human and animal nutrition. Three basic diets were used: "milk alone," an "average American diet" composed of food items as consumed by humans, and a "simulated average American diet" which had the general nutrient composition of the average American diet but was made up, in the main, of a comparatively few human dietary items that might be considered normal components of a rat ration. Three kinds of milk were used: regular (homogenized, vitamin D), VMF and "VMF-4X" (VMF with added nutrients present at four times the usual levels). In additional experiments the effect of iodine supplementation of crude and semi-synthetic rat rations was observed.

MATERIALS AND METHODS

Experimental animals. Albino rats of the Sprague-Dawley strain were obtained from Sprague-Dawley, Inc., Madison, Wisconsin at weaning age (21 days old). Eight male and eight female rats were used per group. They were housed in air-conditioned quarters in screen-bottomed cages. Except for breeding, the animals were kept in individual cages. Except in the "milk alone" experiments, tap water was available to the animals. For the production of first litters on milk alone, the mothers were kept in the regular animal room; a metal chamber with a bed of wood shavings was accessible in each cage. In all subsequent experiments the animals were kept in separate, more secluded quarters which were entered by only a limited number of people. In each cage a two-quart butter crock containing a layer of cellulose bedding material was available to the mother.

For reproduction tests, the rats were mated when the females reached a weight of approximately 200 g. For production of first litters on milk alone, one male was placed in a cage with one female for 2 weeks. Subsequently, for reproduction, one male was

kept with each female until any young produced were 19 to 21 days old. Except for the "milk alone" experiment, first litter young were discarded when they were 3 days old. Subsequent litters were cut back to 6 young per litter at 3 days after birth. These young were sacrificed at weaning except for representative animals from the 2nd litters which were mated when the females reached a weight of approximately 200 g. Some of the 3rd generation animals were raised for 6 weeks after weaning on the diets of their parents.

Diets. "VMF milk" refers to homogenized vitamin D (400 units/quart) milk fortified to bring the total amounts of the indicated nutrients to the following levels (per qt.): 4,000 I.U. vitamin A, 1 mg. thiamine, 2 mg. riboflavin, 10 mg. niacin, 10 mg. iron, and 0.1 mg. iodine. This formula has been used in the production of Gail Borden brand fortified whole milk sold by The Borden Company. "VMF-4X" milk had the added nutrients brought to four times the levels in the VMF milk.

For mineralization the milks were supplemented with $FeSo_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ and $MnSO_4 \cdot H_2O$ to provide 10 mg. iron, 1.5 mg. Cu, and 1.5 mg. Mn per quart.

For the milk-alone and simulated average American diet (S.A.A.D.) feeding, the VMF and VMF-4X milks were prepared three times weekly from regular milk delivered by The Borden Comany plant at Madison, Wisconsin; concentrates used in the dairy industry were employed for the fortification. Control milks always were taken from the same delivery. All milks were stored in the refrigerator. The regular and VMF milks used in preparation of the average American diet were delivered to the American Institute of Baking from The Borden Company plant at Hammond, Indiana. The VMF-4X milk for this diet was prepared by further fortification of the VMF milk.

The average American diet (A.A.D.), formulated by the American Institute of Baking, was based on the U. S. Department of Agriculture Handbook, *Consumption of Food in the U. S., 1909-52 (6)*. It consisted of a wide variety of food items prepared as they might be for human consumption. The diets were made up in their final form at the American Institute of Baking and shipped to the Foundation in the frozen state. Composition of the diet expressed as "per cent of energy" was as follows: fluid milk, 6.7; evaporated milk and cheese, 6.7; eggs, 2.8; bread, 18.9; cornmeal, rice and oatmeal, 3.9; sucrose, 15.4; fats and oils (as such), 20.1; meat, poultry and fish, 12.8; dry beans, peas and nuts, 2.9; potatoes and sweet potatoes, 2.9; vegetables and fruits, 6.5; cocoa, 0.4.

The simulated average American diet was made up of a comparatively few human dietary food items which for the most part might be considered normal constituents of a rat ration. It had approximately the proportions of fat, protein and carbohydrate found in the average American diet in recent years and also furnished levels of vitamins and minerals meeting the recommended allowances for the normal adult human set by the Food and Nutrition Board, National Research Council *(4)*. The proportions of the various food items reflected in a general way Government figures for consumption of those items. Composition of the diet was as follows: enriched white bread, 1,250 g.; 100% whole wheat bread, 312 g.; fresh eggs, 500 g.; canned corned beef hash, 625 g.; oatmeal, 188 g.; crude casein, 270 g.; corn meal, 250 g.; corn starch, 1,330 g.; butter, 300 g.; Crisco, 500 g.; U.S.P. Salts I, 50 g.; Cod liver oil, 15 g.; thiamine, 8 mg.; riboflavin, 10 mg.; niacin, 80 mg. The basal diet was prepared periodically and stored in the frozen state. Each day the proper proportions of milk were incorporated into aliquots of the basal diet in a Waring blender. The milks were added at a level of "1 pint per day" and "1 quart per day"—with 3,000 calories assumed to be one day's ration. The milks were substituted for the entire basal diet on a caloric basis.

For iodine supplementation experiments, a complete semi-synthetic ration containing 4% U.S.P. Salts I and a corn-wheat gluten-brewers' yeast-salts ration *(3)* were supplemented with up to 100 mg. of iodine (as KI) per Kg. of ration. This is roughly 100 times the level of iodine commonly used in rat rations.

Feeding procedures. Except for one paired-feeding test with milk alone, feeding was *ad libitum*. Ample quantities of fresh fluid milk were provided twice daily. The other diets were fed from mid-afternoon to the following mid-morning.

Pathology. All animals were autopsied and tissues from representative animals from each group were examined histopathologically by qualified pathologists.

TABLE 1

Growth at beginning of long term rat tests

Diet	Average weekly gain (1st 6 weeks)	
	Males	*Females*
Regular milk + Fe, Cu, Mn	32.3 g.	23.1 g.
VMF milk + Cu, Mn	34.7	23.3
VMF-4x milk + Cu, Mn	35.7	23.1
Ave. Am. diet with reg. milk	32.3	21.8
Ave. Am. diet with VMF milk	34.5	22.8
Ave. Am. diet with VMF-4X milk	33.4	23.9
S.A.A.D.	20.2	16.0
S.A.A.D. + reg. milk (1 pt. /3,000 cal.)	29.7	20.3
S.A.A.D. + VMF milk (1 pt./3,000 cal.)	31.7	20.9
S.A.A.D. + reg. milk (1 qt./3,000 cal.)	31.9	24.1
S.A.A.D. + VMF milk (1 qt./3,000 cal.)	32.2	24.6
S.A.A.D. + VMF-4X milk (1 qt./3,000 cal.)	32.3	22.6

RESULTS AND DISCUSSION

The rat growth results shown in Table 1 illustrate the well known need for supplementation with iron, copper and manganese *(2)* in order to obtain good growth when rats are raised on milk alone. Growth of male rats was slightly better on the fortified milks plus trace minerals than on the regular milk plus trace minerals. Paired feeding (restriction of food intake to make it equal for all rats) practically eliminated this differential. In the case of the female rats, the growth was practically identical on the various milks if trace minerals were supplemented. Growth on the mineralized regular milk was comparable to that obtained by Bixby *et al. (1)*.

Growth of rats in the first 6 weeks of the long term tests on the average American diet and the simulated average American diet are also shown in Table 1. Good growth was obtained on the former and there was no marked difference in growth rates on the different supplements. Only fair growth was obtained on the S.A.A.D. basal. Marked improvement was obtained with the addition of milk at a level of one pint per 3,000 calories. Only slight additional improvement was observed with the male rats when the level of milk was doubled; however, the female rats definitely grew faster on the higher level of milk.

Reproduction results on milks plus trace minerals and the A.A.D. are given in Table 2. In general, reproduction performance was about the same on the three different "milk-alone" diets. In each case there was at least one instance of difficulty of raising the young to weaning. No satisfactory explanation of this could be arrived at. The loss of a number of the young of the 2nd litters on the Fortified 4X diet just before weaning suggested the possibility of "borderline vitamin E" deficiency *(5)*. One-half of the females in this group were supplemented with vitamin E during the breeding period before giving birth to their 3rd litters. However, as to the 3rd litters, there was no difference between supplemented and unsupplemented animals. Furthermore, tissue examinations and vitamin E assays on the milks did not confirm a vitamin E deficiency.

Reproductive performance on the Average American Diet (Table 2) was quite satisfactory and about the same for the 3 different diets. An

TABLE 2

Reproduction on mineralized milks and average American diet

	Regular	VMF Mineralized milk	VMF-4x
No. of pregnancies			
2nd litters	8	8	8
3rd litters	8	7	8
3rd generation	8	8	8
Live young per litter			
2nd litters	10.7	9.1	9.5
3rd litters	10.8	8.6	7.0
3rd generation	10.0	10.6	10.1
% weaned			
2nd litters	89%	75%	50%
3rd litters	94%	30%	85%
3rd generation	63%	87%	65%
	Average American diet		
No. of pregnancies			
2nd litters	8	7	8
3rd litters	8	8	8
3rd generation	7	6	8
Live young per litter			
2nd litters	8.2	9.4	9.9
3rd litters	11.0	8.9	10.3
3rd generation	8.7	11.0	10.8
% weaned			
2nd litters	97%	93%	97%
3rd litters	91%	97%	90%
3rd generation	64%	67%	100%

exception was the raising of only two-thirds of the 3rd generation rats on regular and fortified milks to weaning while 100% were raised to weaning on the VMF 4X milk.

Results of reproduction tests on the simulated average American diet are shown in Table 3. In the 2nd litters the percentage of young raised to weaning was greater on the milk-supplemented diets than on the basal. All of the 3rd litter young on the fortified milks at 1 qt. per 3,000 calories were raised to weaning. In this experiment, attempts to raise 3rd generation young were uniformly unsuccessful in all of the groups, including those on a control complete synthetic ration, and the detailed results are not recorded herein.

The average weights of the weaned young are given in Table 4. There is a rather consistent tendency toward heavier young at weaning on the fortified milks. Weights of the parent rats at the time they were sacrificed are given in Table 5.

Early growth of the 3rd generation young on the milk plus trace minerals and average American diet is recorded in Table 6. The growth is quite similar to that obtained with the original parent rats at the beginning

TABLE 3
Reproduction on simulated average American diet

Diet	No. live young per litter		% weaned	
	2nd lit.	3rd lit.	2nd lit.	3rd lit.
Basal	8.5	9.2	62	78
Basal (1 pt. reg. milk/3,000 cal.)	9.7	8.0	92	27
Basal (1 pt. VMF milk/3,000 cal.)	9.9	11.8	91	21
Basal (1 qt. reg. milk/3,000 cal.)	8.1	7.0	85	0
Basal (1 qt. VMF milk/3,000 cal.)	10.8	12.0	100	100
Basal (1 pt. VMF-4X milk/3,000 cal.)	9.7	9.0	88	100

TABLE 4
Average weight of weaned young

	2nd litter	3rd litter	3rd generation
Reg. milk + Fe, Cu, Mn	53.0 g.	51.8 g.	41.9 g.
VMF milk + Cu, Mn	50.9	60.8	45.9
VMF-4X milk + Cu, Mn	53.3	60.3	44.1
A.A.D.—reg. milk	45.9	42.3	34.3
A.A.D.—VMF milk	45.9	45.3	34.7
A.A.D.—VMF-4X milk	48.1	46.8	37.5
S.A.A.D.	28.9	34.5	.....
S.A.A.D. (1 pt. reg./3,000 cal.)	48.5	37.7	.....
S.A.A.D. (1 pt. VMF/3,000 cal.)	48.7	43.0	.....
S.A.A.D. (1 qt. reg./3,000 cal.)	54.2	.....	.....
S.A.A.D. (1 qt. VMF/3,000 cal.)	56.0	54.5	.....
S.A.A.D. (1 qt. VMF-4X/3,000 cal.)	49.5	44.7	.....

of the long term trials (Table 1). Animals from each group at 6 weeks after weaning are shown in Figure 1. These selected animals had weights closest to the average for their respective groups.

All animals were autopsied when sacrificed and tissues from representative animals in all groups were subjected to histopathological examination. All animals appeared normal on gross examination, and the few tissue abnormalities observed were of a minor nature and were distributed among control and experimental groups; they were recorded as insignificant by the pathologist.

Spot-checking of feed efficiency, carcass analysis (fat, protein, moisture) and weighing of livers and kidneys on representative animals from each group showed no appreciable effect of the VMF and VMF-4X supplements.

Rats on the iodine supplementation experiment were started at weaning age, at approximately the same weight, and carried on control and iodine-supplemented rations for 13 weeks (see Table 7). The supplement had no marked effect on growth rate. At 13 weeks on experiment, 2 males and 2 females from each group were sacrificed and autopsied; 12 tissues from each of these animals, including thyroid and parathyroid, were subjected to histopathological examination. No abnormalities were found. At 5 months, the remaining males all weighed approximately 500 g. and the females, 280 g. After 9 months, on experiment, the animals were sacrificed and no abnormalities were found on autopsy.

TABLE 5

Average weights of rats at termination

	Males	Females
Mineralized milks (14 mos.)		
Regular + Fe, Cu, Mn	611 g.	377 g.
VMF + Cu, Mn	615	378
VMF-4x + Cu, Mn	649	351
Average American diet (males, 7 mos.; females, 8 mos.)		
Regular milk	525	318
VMF milk	562	340
VMF-4X milk	548	343
Simulated average American diet (9 mos.)		
Basal	531	305
Basal (1 pt. reg. milk/3,000 cal.)	627	390
Basal (1 pt. VMF milk/3,000 cal.)	643	356
Basal (1 qt. reg. milk/3,000 cal.)	668	419
Basal (1 qt. VMF milk/3,000 cal.)	666	423
Basal (1 qt. VMF-4X milk/3,000 cal.)	678	411

TABLE 6

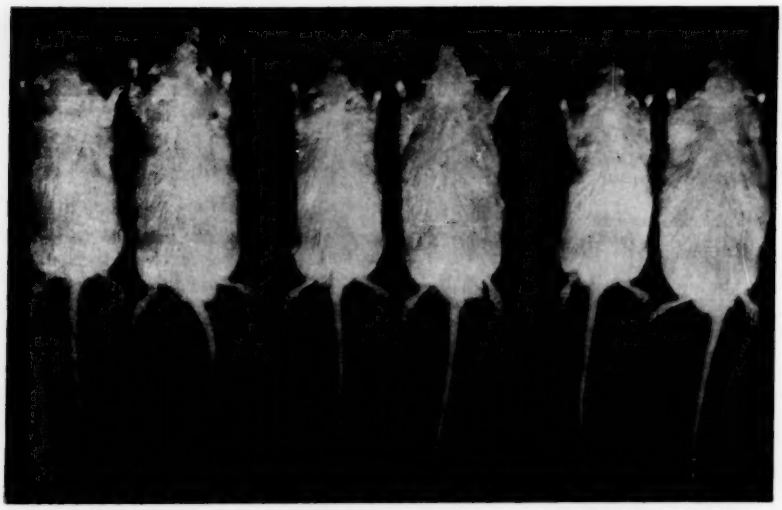
Growth of third generation rats

	Average weekly gain (6 wks.)	
	Males	Females
Regular milk + Fe, Cu, Mn	31.0 g.	21.4 g.
VMF milk + Cu, Mn	29.7	21.2
VMF + 4X milk + Cu, Mn	31.3	23.9
A.A.D. regular milk	32.1	21.5
A.A.D. VMF milk	31.9	21.4
A.A.D. VMF − 4X milk	32.6	20.4

SUMMARY AND CONCLUSIONS

Rats were maintained on the following diets: (a) milk plus trace minerals, (b) average American diet and (c) simulated average American diet. Rats divided into three groups were placed on diets (a) and (b): (1) *control*, (2) *fortified* with vitamins and minerals used in a commercial vitamin and mineral-fortified whole milk, and (3) *fortified to 4 times the usual levels* of these vitamin and mineral supplements. In (a) these milks were the sole source of food, and in (b) the three different milks were incorporated as the fluid milk constituents of the average American diet. The simulated average American diet (c) was supplemented with the regular and fortified milks @ 1 pt./3000 calories and 1 qt./3000 calories. The "fortified 4X" milk was supplemented at 1 qt./3000 calories.

Three litters of rats were produced from groups of eight male and eight female rats placed on each of the diets at weaning age. Rats from the 2nd litters were raised and bred for production of 3rd generation rats. The original parent rats on milks plus trace minerals were kept on experiment for 14 months.



Regular Milk + Fe, Cu, Mn male female | VMF Milk + Cu, Mn male female | VMF-4X Milk + Cu, Mn male female

Figure 1. Representative third generation animals from rats raised on mineralized milk. Each animal was at a weight nearest the average for its own group at 6 weeks after weaning when the picture was taken.

Data on growth, reproduction, feed efficiency, organ weights, carcass analysis and histopathological examination of tissues from representative animals of the various groups did not show any deleterious effects of the vitamin and mineral supplements. In some of the experiments, there was a suggestion of slightly increased growth on the vitamin and mineral supplements (aside from the well known effects of Fe, Cu and Mn in rats on milk alone). In the case of the simulated average American diet, all the milk supplements stimulated the growth rate and growth on 1 qt./3000 calories was somewhat faster than on 1 pt./3000 calories.

In general, reproduction was good although some difficulties were encountered which could not be related directly to the diet.

The over-all conclusion that is drawn from these experiments is that

TABLE 7

Iodine supplementation of rat diets

	Average weights at 13 weeks	
	Males	Females
Synthetic ration	395 g.	228 g.
Synthetic ration + 0.5 mg. I/Kg. ration	385	246
Synthetic ration + 100 mg. I/Kg. ration	394	240
Crude ration	303	211
Crude ration + 10 mg. I/Kg. ration	272	219
Crude ration + 100 mg. I/Kg. ration	323	218

no deleterious effects of the vitamin and mineral supplements were observed; this provides evidence of the dietary safety of the formula under study.

Acknowledgments

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RELATIONSHIP OF AGE WITH STRAIN RETARDATION IN A STARCH GEL

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Wherever linear starch fractions preponderate in food materials, retrogradation (*i.e.*, the parallel association of molecular segments to form crystallites) is a constant occurrence *(11, 16)*. In bread, considerable evidence has accumulated to suggest that the staling process involves primarily a retrogradation of the starch molecules *(1, 7, 17)*. Similarly, problems in the reconstitution of cooked, frozen potatoes *(8)* and in thickening pastes for canning *(3)* indicate the immanence of the retrogradative process.

Studies of starch pastes *(22)* and of bread *(5)* reveal that retrogradation is a time-dependent reaction. In such studies, it has been noted that a characteristic property of staling bread is its augmented toughness on standing. Thus, measures of staleness are often based upon the relative compressibility of the bread crumb *(6, 12, 14)*. In like manner, the "shelf-life" of starch jelly candies is limited by the rate at which induration occurs. The association of hardening with increasing age suggests that a retrogradative process is operative here also. This investigation has been undertaken to ascertain the changes in rheological properties during the aging of such starch jelly candies.

MATERIALS AND METHODS

The starch gel used is identical with that which was described earlier *(19)*. To study the effects of aging, the gel cylinders were removed from the corn-starch molds and wrapped carefully in "Saran Wrap."[a] These samples were stored at room temperature (21° C.) for 2-, 5-, 10-, 30-, and 60-day periods. Data for the one-day sample have been reported already *(19)*. Sufficient material was prepared for several replications of each test period.

Individual sample gels of each chronological group were loaded with 2, 5, 10, 20, 50, 100, and 200 g. weights, respectively, for 20 minutes and then permitted to recover for 20 minutes. No sample was stressed more than once. The manner of derivation of values for strain and corresponding stress was given previously *(19)*.

In order to evaluate the effectiveness of the wrapping material in protecting against moisture loss, moisture determinations were made in the following manner: 3-4 gel cylinders (*ca.* 14-18 g.) were weighed and then blended with 300 g. of water for 5 minutes. During stirring of the slurry, 10 ml. aliquots were placed in tared aluminum moisture dishes. These were weighed and then dried in a 70° C. oven under 29.5" Hg vacuum for 48 hours. After a subsequent weighing, percentage moisture was computed.

RESULTS AND DISCUSSION

When the data are analyzed from the standpoint of the unrecovered deformation (after 20 minutes of recovery), it is found (Figure 1) that as the gel ages, the permanent strain becomes successively smaller. Hence

[a] Dow Chemical Company.

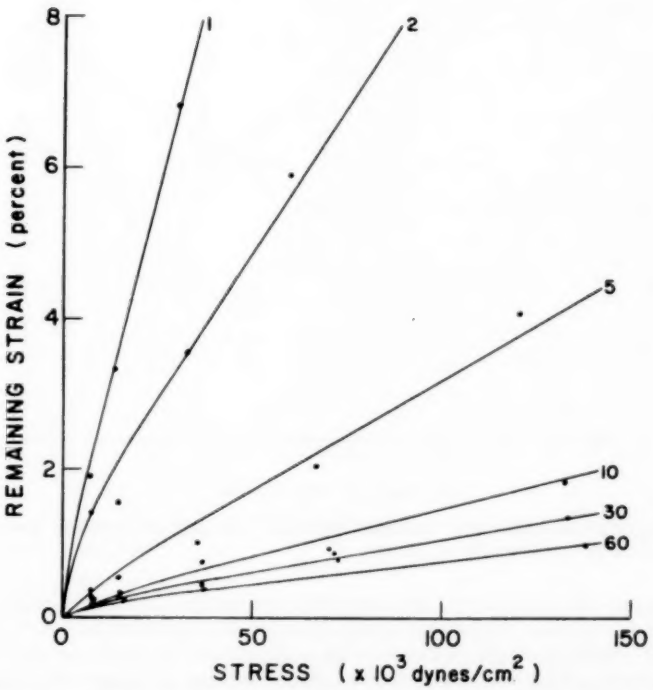

Figure 1. Strain remaining after 20 minutes of loading, followed by 20 minutes of recovery, as a function of amount of stress applied. Age of gel, in days, is indicated for each curve.

it can be concluded that during aging, unattached portions of polysaccharide molecules form associations with their neighbors and are less susceptible to displacement on loading. A continual micellar aggregation likewise can build stronger junctions, which become more and more resistant to rupture. Obversely, with fewer unassociated molecular regions, the amount of crystallization, as a function of stressing, diminishes and consequently contributes less and less to permanent deformation.

It is interesting to note that all the curves of Figure 1 have the same general form: a region concave to the stress axis at lower stresses (*i.e.*, a dilatant condition, as when dextrans are present in honey, *15)* and then a rectilinear relation between stress and remaining strain at higher stresses. If the rectilinear regions of the curves are regarded as representing "viscous flow" *(19)*, their slopes give an estimate of the "viscosity" coefficients of the material. Figure 2 shows the change in such a "viscosity" coefficient as a function of gel age. Although this coefficient is recognizably artificial *(20)*, it does serve to indicate the progressive toughening of the gel.

This increasing coefficient, or resistance to deformation, has a counterpart in the elastic behavior. When the intrinsic elastic modulus of the

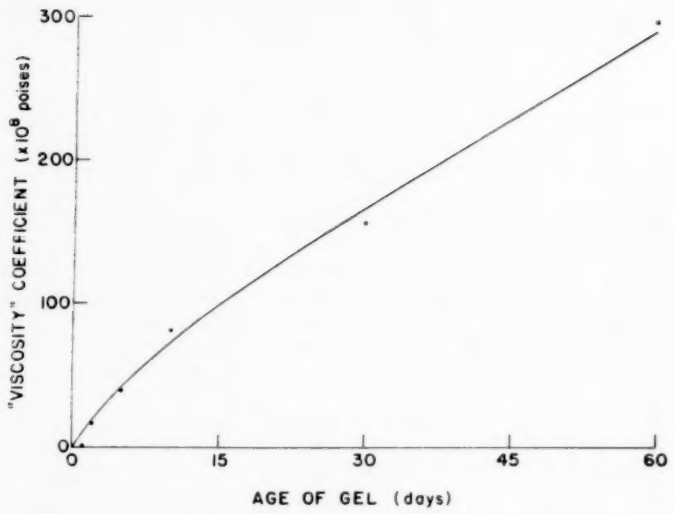

Figure 2. Coefficient of "viscosity" (derived from slope of rectilinear portions of curves of Fig. 1) in relation to gel age.

unstrained gel is derived as indicated previously *(19)*[b], this modulus is found to increase with age (Figure 3). [It is important to recall that a higher elastic modulus indicates that a greater stress must be applied to produce the same amount of strain. Hence a large modulus does not indicate "springiness" *(2, 18)* but rather an opposite quality.]

In Figure 4 are shown the trends of resilient behavior of the starch gel as influenced by its age. A previous analysis of resilience (recovered strain ÷ total strain) in the 1-day gel *(19)* indicated that at lower stresses, displacement of unassociated molecules and parts of molecules as well as molecular straightening predominated. The stress region of $7\text{-}13 \times 10^3$ dynes/cm^2, where the curve of resilience reached a peak, was taken to be the value of the strength of the average micellar bonds. At higher stresses, it was presumed that these bonds are gradually sundered and that crystallization occurs. As the gel ages, there is (a) a progressively greater resilience peak, (b) a continuing increase in the resilience at higher stresses until there is virtually no decrease from the "peak," and (c) a continually higher resilience at lower stresses.

Both the augmentation of the elastic modulus and the changes in resilience with gel age point to the same conclusions derived from the study of unrecovered deformation: increased micellar association with age. This leads to increased average bond strength and hence greater gel rigidity. The initially lower resilience at smaller stresses needs further exploration.

[b] Elastic modulus for any stress = stress ÷ recovered strain. The intercept of the curve of modulus/stress on the modulus axis is taken as the intrinsic modulus of the unstrained material.

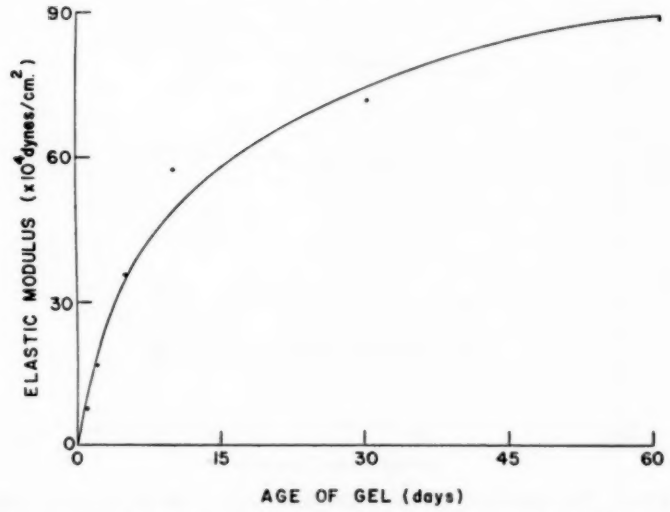

Figure 3. Elastic modulus of the gel (computed on the basis of recovered strain) with respect to gel age.

It would be expected that with more molecular aggregation, the resilience at these lower stresses should approach the peak values. Although a smaller number of free molecular ends exists in the older gel and the existing intermicellar regions are shorter, apparently these can still be affected by the small loads applied.

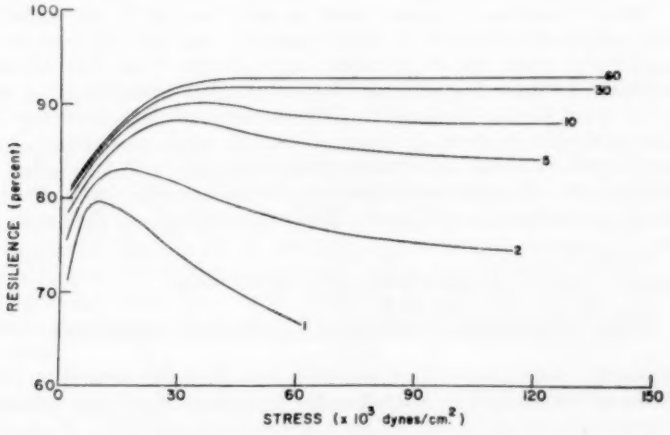

Figure 4. Smoothed curves of per cent resilience (recovered strain ÷ total strain) as a function of stress for different ages of gel. Age of gel, in days, is indicated for each gel.

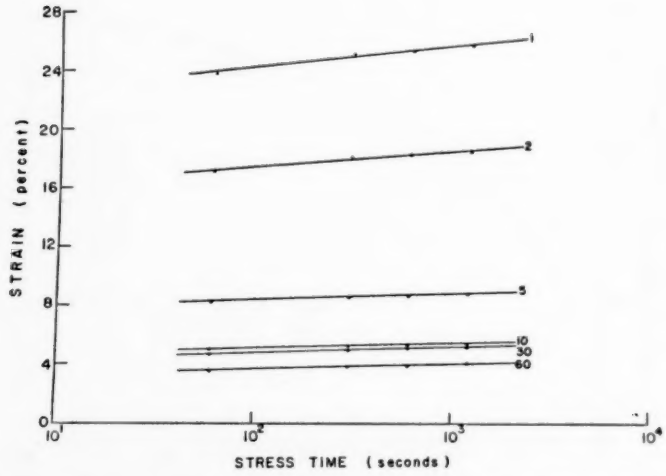

Figure 5. Per cent strain (under 50 gm. load) during time of stress for different ages of gel. Gel age, in days, indicated for each gel. Rectilinear approximations of creep data.

The changing rate of retardation of strain with age may be seen from a comparison of strain/time data for the 50 g. loading (Figure 5).[c] Note that as the gel becomes older, the rate of increase in strain falls off and the total strain attained in 20 minutes is progressively diminished. On the basis of mechanical analogy, these data would indicate a very marked increase in the viscosity of the "dashpot" components of the hypothetical model (see *19*). The time of retardation is greatly decreased. The combined effects of increase in elastic modulus and "viscosity" coefficient with age are indicated in Figure 6, which portrays the relation between the 20-minute total strain for 50 g. loading and gel age. (The distribution of curve slopes of Figure 5 against age follows a similar relationship.) Obviously, the total strain decreases rapidly at first and then begins to level off asymptotically at about 10 days. (Note the close resemblance to the curve of depth of needle penetration against gel age with a gel of similar composition, *10.*) If both coordinates are plotted logarithmically, the curve for the first ten days is rectilinear. This relationship is of the form:

$$\sigma = K\, t^a, \qquad (1)$$

where σ is the total strain attained in 20 minutes,
t is the age of the gel, and
K and a are constants, a representing the slope of the line.

Similarly, rectilinear curves are obtained when the logarithm of the coefficient of "viscosity" is related to the logarithm of gel age (Figure 7)

[c] For convenience, the point distribution for each chronological group in Figure 5 is conceived as being rectilinear.

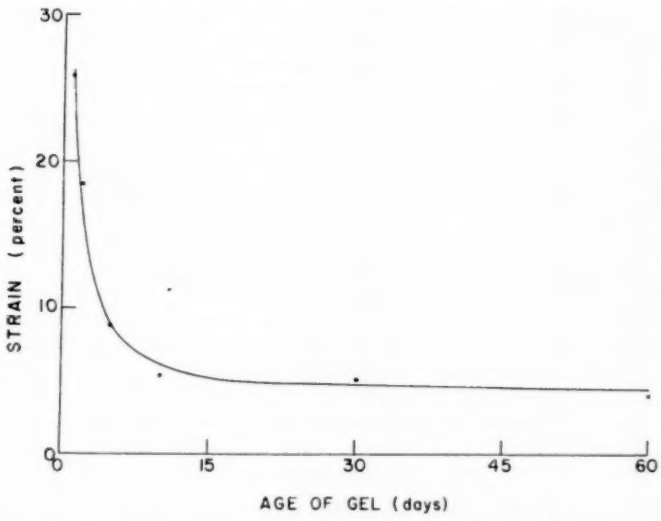

Figure 6. Total per cent strain (under 50 gm. load) after 20 minutes, related to gel age.

and when the elastic modulus is related to the logarithm of gel age (Figure 8). Hence, the form of the "viscosity"/age relationship is:

$$\text{"}\eta\text{"} = K\,t^{a} \qquad (2)$$

where "η" is the coefficient of "viscosity," and the other symbols have the meanings already indicated; and the form of the modulus/age relationship is:

$$n = a \ln t + C, \qquad (3)$$

where n is the modulus of elasticity, C is a constant, and the other symbols have the meanings already indicated.

Although these empirical relationships seem intriguingly simple, their interpretation is not immediately obvious. Mark and Press *(9)* have suggested that the relationship, $\sigma = a \ln t + C$ (as in Figure 5), denotes crystallization during creep. If it be assumed that the amount of crystallization increases with the age of the gel, then a progressive increase in average bond strength will follow the rate at which crystallization occurs. However, although both the elastic modulus and the "viscosity" coefficient depend directly upon bond strength, the quantitative relationship varies in each case.

The loss of moisture from a polysaccharide gel is attended usually by increasing rigidity. Generally, this effect is explained in terms of the replacement of polysaccharide-water bonds by polysaccharide-polysaccharide bonds *(4)*. Hence, if the starch gel in question is losing moisture, any natural cyrstallization tendency will be enhanced. The initial moisture

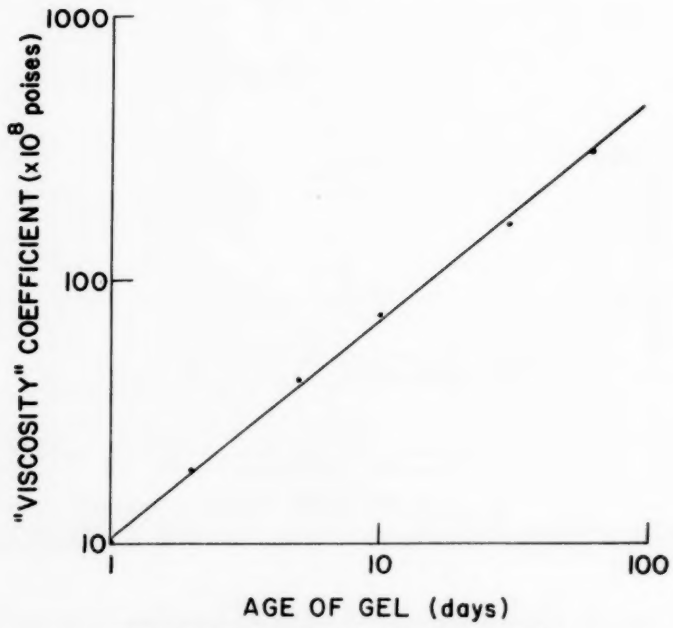

Figure 7. Curve of Figure 2 on logarithmic scale.

content of the one-day-old gel is 22.1%, and it decreases to 21.7% by the 10th day, to 20.8% by the 30th day, and to 18.7% by the 60th day. It appears that the amount of moisture lost here has minimal significance for the retrogradative process in the polysaccharide-water system of the gel. The greater percentage of moisture lost in the second 30 days probably indicates a lowered water-bonding capacity of the gel system, which corroborates the above analysis.

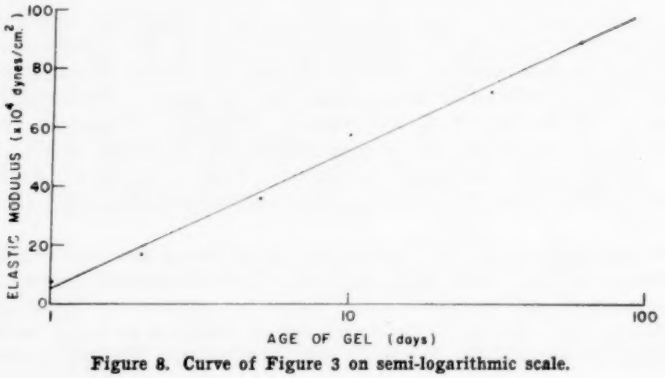

Figure 8. Curve of Figure 3 on semi-logarithmic scale.

Even though the usual measures of crystallinity were not employed in the present study, it can be strongly suggested that the temporal changes in physical behavior found here show the development of crystalline polysaccharide units. For a definitive indication, it will be necessary to measure the changes in soluble starch content, in intensity of X-ray diffraction interferences, *etc. (5, 6, 7, 17)*. Such studies are being pursued.

A technological possibility is also implicit in the results of this investigation. The empirical rectilinear relationships shown above (elastic modulus/logarithm of age; logarithm of "viscosity" coefficient/logarithm of age; and logarithm of total strain/logarithm of age—to 10 days) may be used to diagnose a gel of a particular composition or treatment. Thus, since the slope of the line shows the rate of toughening of the gel, very long term tests may not be necessary to ascertain whether particular aspects of composition or treatment are effective in increasing "shelf-life." Quite possibly penetrometer information, such as that obtained by Martin *(10)*, could be treated in this fashion. It has been indicated *(13, 21)* that penetrometer data may be interpreted in terms of "viscosity."

SUMMARY

Creep and recovery measurements were made on a starch gel at different ages and with varying loads. The modulus of elasticity and coefficient of "viscosity" increased with gel age, while total strain attained and rate of retardation of strain decreased. Resilience of the gel increased as time of aging increased. Since little moisture loss occurred in storage, the above effects indicated an intrinsic retrogradation of the polysaccharide molecules, *i.e.*, increased parallel association at micellar junction points, to strengthen the network in the gel.

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ON THE STRUCTURE OF "HYDROXY-α-CAROTENE" FROM ORANGE JUICE

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In an examination of the carotenoids of Valencia orange juice, an hydroxy-α-carotene-like substance was found *(1)* as a minor constituent occurring to the extent of around 2 to 3% of the total carotenoids; similar fractions have also been obtained from orange peel *(4)*, tangerines (pulp and peel), Ruby red grapefruit (pulp and peel), peaches and apricots *(2)*, in amounts of 1% or less of the total carotenoids. Similar substances had been obtained previously by other workers from several sources, including *Mycobacterium phlei (6)*, leaves *(13)*, yellow corn grain *(15)*, and corn seedlings *(10)*. It appears that "hydroxy-α-carotene" is a widespread minor carotenoid constituent, but a sufficiently rich source has not yet been found so that the substance could be obtained in crystalline form. Hydroxy-α-carotene is not discussed in the monographs of Karrer and Jucker *(7)* or Goodwin *(5)*; the only mention is a reference to its occurrence in *Zea mays* in the former. The present communication gives further information on "hydroxy-α-carotene," including indirect evidence indicating that it is not a provitamin A.

EXPERIMENTAL METHODS

The experimental methods used in countercurrent distribution and chromatography were previously described *(1)*.

Iodine treatment. Ten ml. of a carotenoid solution in petroleum ether (or benzene) having an adsorbance at the principal maximum of around 1.5, measured in a one-cm. cell in a Cary recording spectrophotometer, was treated with 0.2 ml. of a 0.001% solution of iodine in petroleum ether (or benzene) and then exposed to light from a daylight fluorescent lamp at approximately 260 foot candles, for 30 minutes. Spectral adsorption curves were then run within a few minutes.

Acid treatment. Carotenoid solutions were evaporated in vacuo and the residue dissolved in a solution of hydrochloric acid in methanol (1 volume of concentrated acid plus 9 volumes of methanol). After standing at room temperature for 10 minutes, an excess of potassium hydroxide in methanol was added, and the solution worked up as previously described *(3)*. The product was rechromatographed and spectral absorption curves were run on the band or bands.

RESULTS AND DISCUSSION

The "hydroxy-α-carotene" from orange juice *(1)* was obtained on countercurrent distribution of the saponified carotenoids (unsaponifiable fraction) with the system petroleum ether—99% methanol in the same fraction as cryptoxanthin (3-hydroxy-β-carotene), indicating the presence of one hydroxyl group and the probable absence of other oxygen atoms such as cyclic ether groups which would change the distribution coefficient. On chromatography on columns of magnesia (Westvaco No. 2642) plus filter aid, and eluting with a solution of 0.5% ethanol in petroleum ether,

"hydroxy-α-carotene" moved down the column more rapidly than cryptoxanthin, and readily separated from it in a manner very similar to that in which α-carotene separates from β-carotene and lutein (3,3′-dihydroxy-α-carotene) separates from zeaxanthin (3,3′-dihydroxy-β-carotene), using appropriate eluents. Mixed chromatograms of "hydroxy-α-carotene" from orange juice and from leaves and corn oil showed no separation, indicating their probable identity. "Hydroxy-α-carotene" fractions as obtained by countercurrent distribution followed by chromatography usually contain considerable amounts of colorless, readily crystallizable material, apparently sterols. The presence of these substances may also be a factor in the failure thus far to obtain this material in crystalline form.

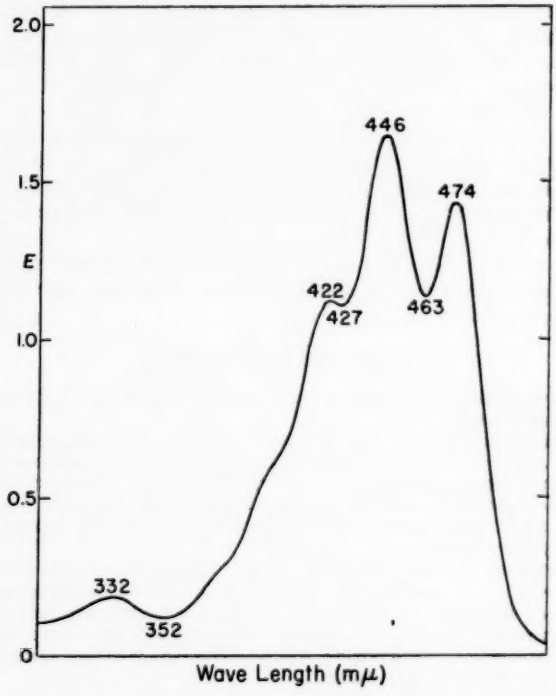

Figure 1. Spectral absorption curve of "hydroxy-α-carotene" (petroleum ether).

The spectral absorption curve of "hydroxy-α-carotene" (Figure 1) is indistinguishable from those of α-carotene or lutein in the same solvent. Therefore, all three must contain the same conjugated double bond system. Based on the spectral absorption maxima, there are a number of possible structures of "hydroxy-α-carotene," including a monocis-cryptoxanthin, cryptoxanthin epoxide, and α-carotene (Figure 2) with an hydroxyl group substituted on the 3 or 4 carbon of the β-ionone ring, or the 3′ carbon of the α-ionone ring. Naturally occurring carotenoids containing hydroxyl

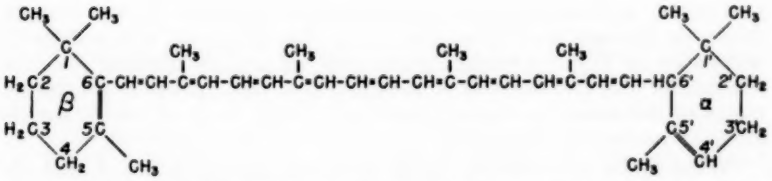

Figure 2. Structural formula of α-carotene (β and α in rings indicate β- and α-ionone rings, respectively).

groups substituted on the 2-carbon of either ring or on any of the methyl groups are unknown.

Behavior on iodine treatment. The spectral absorption curve (Figure 1) of "hydroxy-α-carotene" does not resemble that of a cis-isomer of cryptoxanthin. Treatment with iodine in light confirms the nonidentity of the two substances (Table 1). With all-trans α-carotene, β-carotene, cryptoxanthin or lutein, iodine treatment resulted in a decrease of 2-3 mμ in the wavelength of the two principal maxima; the same was true of "hydroxy-α-carotene," indicating it to be an all-trans compound. On similar treatment of a cis-cryptoxanthin fraction which had absorption maxima close to those of "hydroxy-α-carotene," little change was observed; the isomerized mixture had absorption maxima almost identical with those of isomerized all-trans cryptoxanthin.

On chromatography of the monol fraction a band is sometimes obtained just below "hydroxy-α-carotene" on the column with principal absorption maxima 5 mμ lower in wave length. The absorption maxima of this substance after iodine treatment were very close to those of iodine treated "hydroxy-α-carotene" (Table 1), indicating this substance to be a monocis "hydroxy-α-carotene."

Effect of acid on "Hydroxy-α-Carotene." Cryptoxanthin epoxide, which has the same conjugated double band system as α-carotene, on treatment with acid is isomerized to cryptoflavin *(8)* (cryptoxanthin furanoxide), with a resulting shift in the absorption maxima of about 20 mμ toward shorter wave lengths. "Hydroxy-α-carotene" on acid treatment was recovered essentially unchanged, showing it was not cryptoxanthin epoxide.

Effect of acid on lutein. On treatment with acid, lutein was reported

TABLE 1

Effect of iodine in light on "hydroxy-α-carotene" and related substances

Component	Solvent	Absorption maxima (mμ)	
		Original	Iodine treated
"Hydroxy-α-carotene"	PE	474,446,422	471,443,(419)
Cis-"Hydroxy-α-carotene"	PE	469,441,(418)	471,442,419
α-carotene	PE	474,446,422	472,443,420
β-carotene	PE	477,450,(426)	474,448,(426)
Cryptoxanthin	PE	478,451 (426)	474,447 (424)
Cis-cryptoxanthin	PE	473,447,(425)	(474),447,(425)
Lutein	B	487,457,434	484,455,(430)

PE = petroleum ether; B = benzene; figures in parentheses are approximate values for humps or shelves on spectral absorption curves.

(12, 13, 16) to form an hydroxy-α-carotene-like substance. It was recently shown by Petracek and Zechmeister *(11)* that treatment of a carotenoid containing an allylic hydroxyl group with an acid in methanol solution resulted in the formation of a methyl ether. This reaction provides a means of differentiating between hydroxyl groups on the 3- and 3′-carbon atoms in α-carotene (Figure 2).

When lutein was treated with hydrochloric acid in methanol, and the recovered carotenoids subjected to countercurrent distribution in a Craig apparatus with the system petroleum ether–99% methanol *(1)*, two fractions were obtained with N_{100} values (tube number of maximum calculated per 100 transfers) of 10 (unchanged lutein, 14%) and 38 (85%), respectively. The value of 38 is intermediate between those of lutein (11) and "hydroxy-α-carotene" (57); it is in close agreement with values of 38-39 previously obtained *(4)* for monoether (epoxide and furanoxide) monol fractions. This indicates that the main fraction above is the 3′-methyl ether of lutein.

After chromatography the main product had spectral absorption maxima in petroleum ether of 473,445 and 422 mμ, in good agreement with the values for lutein and hydroxy-α-carotene. A mixed chromotogram with "hydroxy-α-carotene" separated readily, the ether being the upper band; with cryptoxanthin the ether was the lower band. Lutein requires a much stronger eluent than does cryptoxanthin for ready elution (3.5% and 1% ethanol in petroleum ether, respectively).

Under the conditions used, most of the lutein was converted to the monomethyl ether which has a similar spectrophotometer curve to that of lutein, but which differs from lutein considerably in behavior on chromatography and countercurrent distribution. When zeaxanthin and "hydroxy-α-carotene" were treated similarly with hydrochloric acid in methanol, both were recovered essentially unchanged. The hydroxyl groups in zeaxanthin are on the 3-carbons of β-ionone rings, hence the hydroxyl group in "hydroxy-α-carotene" is also probably in this position and definitely not on the 4-carbon of the β-ionone ring or the 3′-carbon of the α-ionone ring.

"Hydroxy-α-carotene" apparently has an α-ionone ring at one end of the molecule and a 3-hydroxy β-ionone ring at the other. A substance with this structure would not be a provitamin A. Carotenoids having an unsubstituted β-ionone at one or both ends of the molecule have vitamin A activity; the α-ionone ring and the 3-hydroxy β-ionone ring are both inactive.

SUMMARY

Hydroxy-α-carotene-like substances occur rather widely in small amounts in various plant materials such as oranges, peaches, and leaves. The "hydroxy-α-carotene" from orange juice has the probable structure 3-hydroxy-α-carotene in which the hydroxyl group is on the β-ionone ring. A substance with this assumed structure would not be a provitamin A.

Acknowledgment

Spectrophotometer data reported here were obtained by Glen F. Bailey.

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ERRATA, VOLUME 21, 1956

Food Research, **20**, 666-686 (1955) *(Ball and Olson: Foundation of Food Process Calculation Methods.)

The twelfth line on page 682 reads:

shell) can be shown, by solid geometry, to be as indicated in eq. (2).

The twelfth line on page 682 should read:

shell) can be shown, by solid geometry, to be roughly as indicated in eq. (2).

In equation (6) and in the third line from bottom of page 682, the symbol v_a should be replaced by the symbol v_o.

Referring to the fifteenth line on page 683, the following footnote is to be added:

* Equations (2), (3), and (6) are very rough approximations of the relationships indicated. They are in greatly simplified form, as compared to equations which express the relationships precisely. While the values in Table 1 are not correct, they are in approximately the same relative orders of magnitude as the correct values. Since these values provide the intended substantiation to the principle under consideration and thus adequately serve the purpose of this discussion, it was decided that the equations in simplified form should be used.

Caption of Table 1, page 683 reads:

Ratios of radial wall thickness of the peripheral shell to radial wall thickness of shell "*a*" when the radius of shell "*a*" equals 0.01 inch in the No. 10 can ($l = 7; r = 3.1$).

Caption of Table 1 should read:

Ratios of radial wall thickness of shell "*a*" to radial wall thickness of the peripheral shell when the radius of shell "*a*" equals 0.01 inch in the No. 10 can ($l = 7; r = 3.1$).

Heading of columns 2 and 4 of Table 1 reads:

Ratio
$\triangle x_p / \triangle x_a$

Heading of columns 2 and 4 of Table 1 should read:

Ratio
$\triangle x_a / \triangle x_p$

The fourth line from bottom of page 683 reads:

a No. 10 can were desired, the ratio of $\triangle x_p$ to $\triangle x_a$ would be 95,700.

The fourth line from bottom of page 683 should read:

a No. 10 can were desired, the ratio of $\triangle x_a$ to $\triangle x_p$ would be 95,700.

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* Reported in 1956.

DIFCO

Bacto-Dehydrated Media for Microbiological Assay of VITAMINS and AMINO ACIDS

These media contain all the necessary nutriments for the growth of specified test organisms for the microbiological assay of vitamins and amino acids except for the component under assay. These basal media require only the addition of specified increasing amounts of the vitamin or amino acid being assayed to obtain a growth response which may be measured by acidimetric or turbidimetric methods for the construction of standard curves. The vitamin or amino acid content of the material under assay is determined by adding appropriate concentrations of the test substance to the basal medium and comparing the growth response obtained with that of the standard.

BACTO-RIBOFLAVIN ASSAY MEDIUM
BACTO-NIACIN ASSAY MEDIUM
BACTO-THIAMIN ASSAY MEDIUM
BACTO-PANTOTHENATE ASSAY MEDIUM
BACTO-B_{12} ASSAY MEDIUM USP
BACTO-CS VITAMIN B_{12} AGAR
BACTO-FOLIC ACID ASSAY MEDIUM
BACTO-PYRIDOXINE ASSAY MEDIUM
BACTO-BIOTIN ASSAY MEDIUM
BACTO-CHOLINE ASSAY MEDIUM
BACTO-CF ASSAY MEDIUM
BACTO-TRYPTOPHANE ASSAY MEDIUM
BACTO-LEUCINE ASSAY MEDIUM
BACTO-METHIONINE ASSAY MEDIUM
BACTO-LYSINE ASSAY MEDIUM
BACTO-ISOLEUCINE ASSAY MEDIUM
BACTO-ARGININE ASSAY MEDIUM
BACTO-TYROSINE ASSAY MEDIUM
BACTO-CYSTINE ASSAY MEDIUM

The method employed in carrying stock cultures of the test organisms and preparing the inoculum for microbiological assay is important. The following media have been developed especially for carrying stock cultures and for preparation of the inoculum:

BACTO-MICRO ASSAY CULTURE AGAR
BACTO-MICRO INOCULUM BROTH
BACTO-B_{12} CULTURE AGAR USP
BACTO-B_{12} INOCULUM BROTH USP
BACTO-NEUROSPORA CULTURE AGAR

BACTO-VITAMIN FREE CASAMINO ACIDS, dehydrated, is an acid hydrolysate of vitamin free casein prepared especially for laboratories investigating microbiological assay of vitamins.

Descriptive literature available upon request

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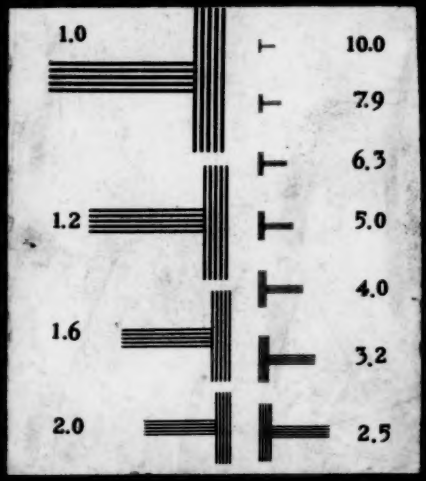
RESOLUTION CHART



100 MILLIMETERS

INSTRUCTIONS Resolution is expressed in terms of the lines per millimeter recorded by a particular film under specified conditions. Numerals in chart indicate the number of lines per millimeter in adjacent "T-shaped" groupings.

In microfilming, it is necessary to determine the reduction ratio and multiply the number of lines in the chart by this value to find the number of lines recorded by the film. As an aid in determining the reduction ratio, the line above is 100 millimeters in length. Measuring this line in the film image and dividing the length into 100 gives the reduction ratio. Example: the line is 20 mm. long in the film image, and $100/20 = 5$.

Examine "T-shaped" line groupings in the film with microscope, and note the number adjacent to finest lines recorded sharply and distinctly. Multiply this number by the reduction factor to obtain resolving power in lines per millimeter. Example: 7.9 group of lines is clearly recorded while lines in the 10.0 group are not distinctly separated. Reduction ratio is 5, and $7.9 \times 5 = 39.5$ lines per millimeter recorded satisfactorily. $10.0 \times 5 = 50$ lines per millimeter which are not recorded satisfactorily. Under the particular conditions, maximum resolution is between 39.5 and 50 lines per millimeter.

Resolution, as measured on the film, is a test of the entire photographic system, including lens, exposure, processing, and other factors. These rarely utilize maximum resolution of the film. Vibrations during exposure, lack of critical focus, and exposures yielding very dense negatives are to be avoided.